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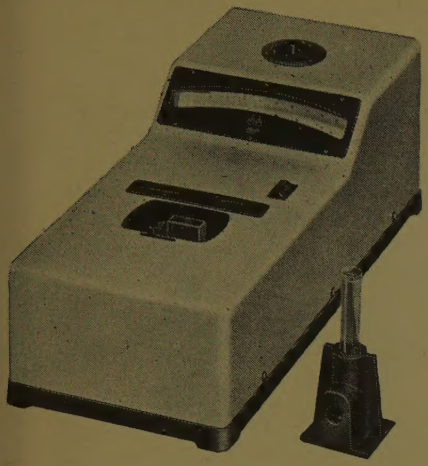

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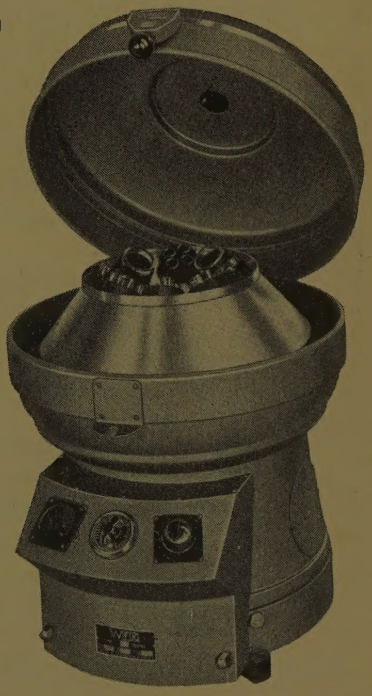

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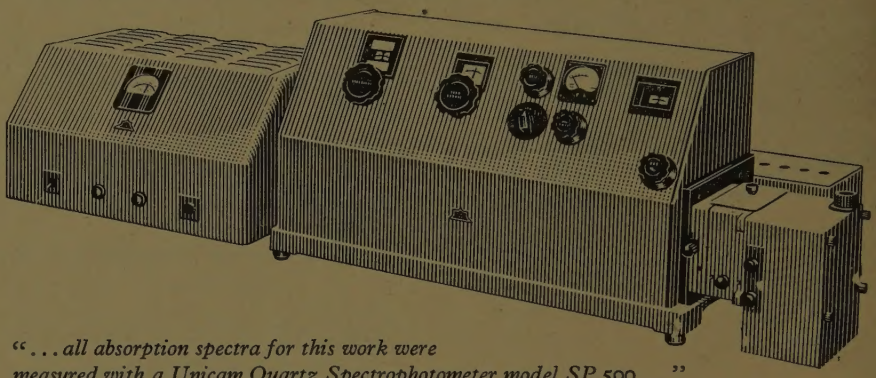

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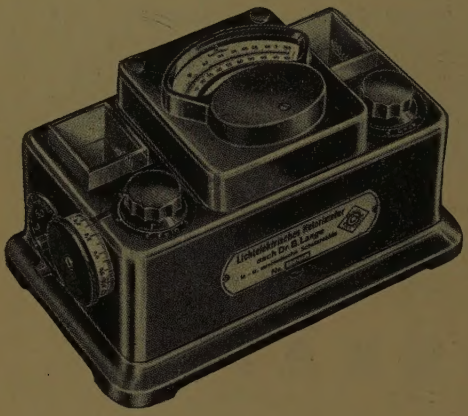

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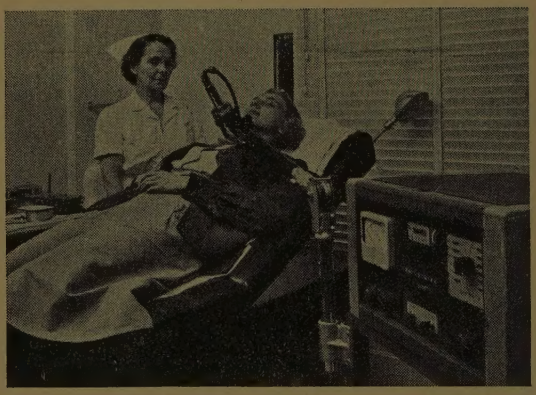

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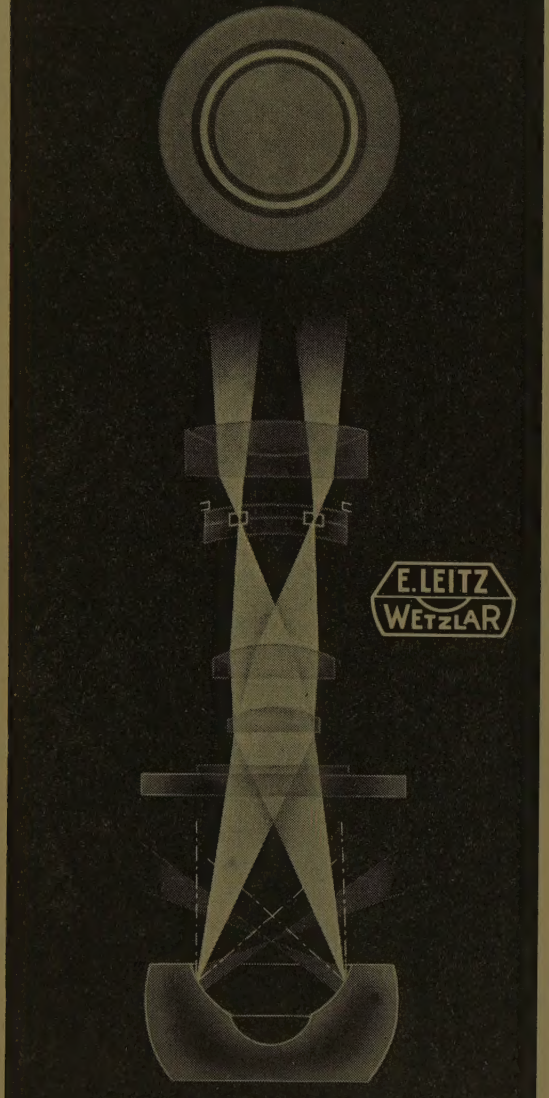


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# THE EFFECT OF ANEURIN ON UTERINE CONTRACTIONS

By S. TIMONEN and K. A. SCHRODERUS

*From the First Women's Clinic of the University of Helsinki, Finland*

(Received for publication Sept. 20, 1952)

Nervous stimulus, passing from the nerve-dings into the muscle fibres, needs an etyl compound, acetylcholine, as humoral nveyor. Stimulation of the parasympathetic rvous system causes acetylization of the oline into acetylcholine; the latter having 1000 to 100 000 times more powerful effect the muscle fibres. The enzyme that cata-zes the process is unknown. The inverse action, the splitting of acetylcholine into oline and acetic acid, is brought about by olinesterase. The action of this enzyme n be inhibited by physostigmine prepara-ns. The well-known effect of prostigmine omoting uterine tonus and contractions is e to this prevention of the splitting of etylcholine. If aneurin, as frequently clai-ed (e. g. Kinnunen, 1946), generally par-ipates in physiological processes of ace-ization, it should produce an essential ect wherever acetylcholine exercises its rasympathetic activity, i. e. in the functions the intestine, urethra, bladder, heart, ner-us system and uterus. Disturbances in the eurin metabolism might lead to exhaustion and inertia of the uterus during labour since the body does not contain any stored aneurin.

As early as in 1912 Ogata observed that patients suffering from beri-beri exhibited uterine inertia and atonia, as well as post partum bleedings and endometritis to a higher degree than normally in puerperium.

Stähler (1937) considered aneurin to have a favorable effect in cases of uterine inertia. He assumed that the $B_1$-vitamin sensitized the uterine muscle in some way. In fact he warned against the use of large aneurin doses towards the end of pregnancy, since they might induce premature labor. Similar observations have been made by Kleine (1940) and Rochat (1940). On the other hand, Hildebrandt (1940) and Neuweiler (1940) were unable to demonstrate any clear effect in cases of uterine inertia. The latter author registered the contractions using Frey's tocograph.

Hashimoto (1937) demonstrated that extract of aneurin exercised a tonus increasing effect on the isolated uterus of a rat

and promoted automatic contractions. Pregnant and puerperal uteri were more sensitive. Using suitable dosages, he was able to increase intensively the sensitivity to the drug. Chemov & Mazaev (1947) succeeded in 25 to 50 per cent of their animal experiments in increasing the uterine tonus and intensifying the contractions through administration of vitamin $B_1$.

Several investigators have found that a clear correlation exists between aneurin and acetylcholine, so that the former increases the effect of the latter. It has been assumed that aneurin, as well as physostigmine, inhibits the action of cholinesterase, the influence of vitamin $B_1$ being cumulative. It is of interest to notice, in this connection, that the level of serum cholinesterase is low in women of the childbearing age, being particularly low during pregnancy (Zeller, Birkhäuser, Wattenwyl, Wenner).

Lipman (1946) has proved that the same enzyme catalyses acetylization of both the sulfonamides and of choline.

## MATERIAL AND METHODS

We have studied the effect of aneurin on the pains during labor. The experiments were carried out at the First Women's Clinic, on 47 patients showing signs of uterine inertia and exhaustion.

In the course of these experiments, we made intravenous administrations of 100 mg aneurin and recorded the pains. For the registration we used in 1947—1948 a tocometer of our own construction, functioning with the aid of air-pressure, and in 1949—1950 Lorand's tocograph.

## RESULTS

If we examine Table I, attention is directe in the first instance, to the sharp differen in the effects of aneurin in uterine inert and uterine exhaustion.

Among 25 cases of the former group, remained unaffected in regard to the strengt of the contractions, in 8 cases a slight, a in 2 cases a considerable, effect was foun Of the 22 cases in the exhaustion group on 2 remained completely unchanged, 8 reveale slight changes, 10 a considerable improv ment, and 2 were markedly affected. If it assumed that aneurin plays an importa part in the enzymatic process of choli acetylizing, the difference between the groups becomes comprehensible. The ro of the parasympathetic nervous system uterine contractions can schematically divided into three stages.

1. Stimulus affecting the synapsis throug a nervous-hormonal activity.
2. Synaptic capability of responding to t stimulus by acetylcholine formation.
3. Capacity of the muscle fibre to contra under the influence of acetylcholine.

If the nervous-hormonal activity is d ficient, we have a case of inertia. Th deficiency need not affect the acetylizi capacity of the synapsis, nor the sensitivi in the muscular reaction, and therefo therapeutic measures directed against the do not elicit any response. Since the effe of aneurin has been previously studied cli cally, predominantly in regard to inertia, is not surprising that the results are contr dictory. In animal experiments, as well prior to a spontaneous onset of labor, a cor sponding state of inertia prevails. T

## Table I

Table I shows the effect of the aneurin injection on the uterus in labor. The signs are: — negative effect or no effect at all, ± dubious effect, + slightly affected, ++ considerably affected, +++ markedly affected.

| Inertia uteri | | | | | | Exhaustio uteri | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Number of cases | Age | Number of births | Uterine reaction | | | Number of cases | Age | Number of births | Uterine reaction | | |
| | | | Tonus | Interval of contractions | Strength of contractions | | | | Tonus | Interval of contractions | Strength of contractions |
| 1 | 23 | IV | + | − | − | 26 | 24 | II | ± | − | ++ |
| 2 | 32 | II | ± | ± | + | 27 | 31 | I | | + | + |
| 3 | 28 | I | − | ± | ± | 28 | 29 | I | | − | ++ |
| 4 | 32 | I | | ± | + | 29 | 39 | I | | − | + |
| 5 | 40 | I | | ± | ± | 30 | 24 | I | | − | + |
| 6 | 25 | I | | − | ± | 31 | 29 | II | ± | + | + |
| 7 | 30 | I | ± | ± | ± | 32 | 37 | III | ± | − | − |
| 8 | 33 | I | ± | ± | ± | 33 | 24 | I | | − | ++ |
| 9 | 21 | I | − | + | ++ | 34 | 28 | I | | ± | + |
| 10 | 30 | VII | ± | ± | ± | 35 | 24 | I | | ± | − |
| 11 | 22 | I | ± | ++ | + | 36 | 29 | II | ± | + | +++ |
| 12 | 36 | VI | ± | ± | ± | 37 | 20 | I | − | ± | ++ |
| 13 | 39 | I | − | ± | ± | 38 | 23 | I | − | ± | +++ |
| 14 | 26 | I | − | − | ± | 39 | 34 | I | + | − | ++ |
| 15 | 33 | I | ± | ± | ± | 40 | 29 | I | − | − | ++ |
| 16 | 24 | III | ± | ± | ± | 41 | 26 | I | − | − | ++ |
| 17 | 21 | I | − | − | ± | 42 | 34 | II | − | ± | + |
| 18 | 19 | I | + | − | + | 43 | 23 | I | − | ± | ++ |
| 19 | 33 | II | | ± | + | 44 | 28 | VI | ± | − | + |
| 20 | 28 | I | | ± | + | 45 | 27 | I | ± | − | + |
| 21 | 33 | I | | − | + | 46 | 30 | I | ± | − | ++ |
| 22 | 34 | I | − | − | + | 47 | 30 | IV | ± | − | ++ |
| 23 | 24 | I | ± | + | ± | | | | | | |
| 24 | 24 | II | − | − | ± | | | | | | |
| 25 | 29 | II | + | + | ++ | | | | | | |

owerful increase of sensitivity to reaction« ought about by Hashimoto in animal periments might be interpreted as follows. injecting the posterior lobe hormone he t an end to the state of inertia and was ly then enabled to study the effect of eurin: in fact several patients belonging the inertia group revealed clear indications hormonal weakness in their menstrual story and constitution.

On the other hand, the weak point of the haustion group seems to lie in the synaptic pacity to respond to stimulus, *i. e.* in the paralysation of the fermenting process required for acetylization. Aneurin administered in large doses seems to eliminate this weakness. Theoretically there is a great difference between hypovitaminosis and hypervitaminosis. Disturbances in the aneurin metabolism have been found in association with pregnancy and parturition (Pitkänen, Hildebrandt, Neuweiler etc.). If we consider the large amounts and high concentrations required in order to obtain any effect, we are still inclined to assume that this is not a matter of elimination of hypo-

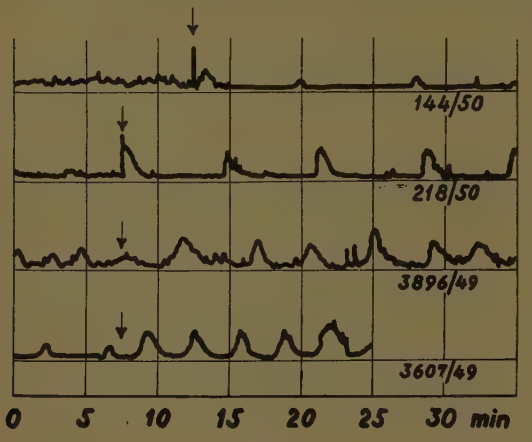


Fig. 1.

Figure 1 shows some typical records registered with the Lorand's Tocograph. The arrow indicates the injection of aneurin. The record marked 144/50 refers to case No. 11 in Table I in the uterine inertia group. The record 218/50 refers to case No. 43, 3896/49 to case No. 36 and 3607/49 to case No. 46 of the uterine exhaustion group.

vitaminosis, a peculiar effect of overdosage. Such an indication is also provided by Sysi's (1945) observation that the near point of the eye can be brought closer for some time by an aneurin injection in 83 per cent of the cases. He did not observe any correlation between this phenomenon and aneurin deficiency. Here we have a similar hypervitaminatic effect on the accomodation muscles as on the uterine muscle described above. The utrine tonus in the two groups did not differ noticeably in regard to the effect of an injection. In 11 cases the tonus was lowered after injection, in 4 it was increased and in 17 no change could be observed. In 15 cases registered with the tocometer the uterine tonus could not be measured. At least, in those cases where the tonus showed a considerable rise in the beginning of the experiment, there was a regular weaking after the injection. The irregular spasticity between the contractions was in many case diminished. The intervals between the pain remained unaltered in inertia as well as i exhaustion.

In 21 cases the interval between pains wa shortened, in 7 prolonged, and in 19 n change could be observed. The latency time before the maximum was reached, varie greatly, and similarly the duration of th effect of one injection showed great varia tion. The effect of a renewed aneurin injec tion, after 30—60 minutes, was weaker tha that of the first.

The conclusion can be drawn from thes tests that, in pure cases of exhaustion aneurin therapy can promote the uterin activity, a fact which, in addition to theoretical significance, can sometimes als be of practical value. In inertia, on the othe hand, it seems that no more can be don than to eliminate the effect of exhaustio frequently associated with it.

## SUMMARY

1. The effect of aneurin on inadequat pains in 47 cases has been studied. An injec tion of 100 mg was administered and th results were recorded tocographically.

2. In cases of inertia no clear effect coul be observed.

3. In cases of exhaustion the strength uterine contractions was increased as a rul sometimes to such an extent that there wa a therapeutical value of the procedure.

4. The effect is ascribed by the write to the circumstance that aneurin promot the acetylization of choline, thus renderi the synaptic reaction more sensitive. It suggested that the phenomenon is large due to hypervitaminosis produced by lar doses of vitamin $B_1$.

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# SEPARATION OF PORPHYRINS BY MEANS OF COUNTER-CURRENT DISTRIBUTION

By K. G. PAUL

*From Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden*

(Received for publication Dec. 5, 1952)

The components in a mixture of porphyrins can be separated from each other in several ways: By the use of their different solubilities in organic solvents or by adsorption or partition chromatography of the free porphyrins or their methyl esters. In most cases these methods separate the porphyrins according to the number of carboxyl groups.

Separation can also be achieved by means of partition between an acid, aqueous phase and some solvent, immiscible with water (Willstätter & Mieg, 1906; Zeile & Rau, 1937). An approach to a cationic separation of porphyrins by adsorption chromatography has been made (Lucas & Orten, 1951). As yet no combination of solvents has been found for cationic separation with paper chromatography.

A simple microscale method is, however, highly needed for the separation of porphyrins as cations. For some time we have been using a combination of porphyrin partition in the system organic solvent / dilute acid or acid buffer with the counter-current distribution technique as designed by Craig (Gregory & Craig, 1951). We have found it to offer certain definite advantages over the common procedure with repeated extractions in a separatory funnel as a test for purity. When the separation is made in an all-glass apparatus or in a series of gla tubes, as described in the sequel, it is po sible to estimate at an early stage if a su able pair of solvents had been chosen, essentially a question of the acidity of t buffer. At the end of the separation illumination of the whole series of fractio with an ultraviolet lamp will immediate disclose inhomogeneities. In the spectr photometric analysis the fractions are co pared in absolutely identical solvents, whi is of utmost importance for their spectr identification. No material is lost. Even su small quantities of *e. g.* proto- and copr porphyrins as those obtainable from 5 ml blood can easily be handled and furth analyzed by paper chromatography. Final with a sufficient number of transfers it possible to compare the experimental poi with the distribution curve, calculated fo pure substance.

## EQUIPMENT AND PROCEDURE

An all-glass counter-current distribution ap ratus is, of course, the instrument to be p ferred, if available. However, sometimes even few transfers give sufficient information. In su cases the following equipment has been found be quite satisfying. A series of glass tubes w ground-in glass stoppers is used, or, in the abse of chloroform, ordinary 10 × 100 mm centrif

s with rubber stoppers. Equal volumes ($a$ ml) he organic solvent and the buffer or the acid, iously mutually saturated in a separatory fun- are pipetted into the first tube (tube 0). The tance to be analyzed is added, either in the l state, if easily dissolved, or in a *small* volume drop) of either solvent. Sometimes it is pre- ble to dissolve the sample in the acid or alkaline ponent of the buffer, add the other component, then an amount of the organic solvent just igh to saturate the buffer with it.

o each of the other tubes in the series are n $a$ ml of the organic solvent. Tube 0 is en, the two phases allowed to separate, and the om layer transferred to the next tube (tube 1) a dropping pipette. Another $a$ ml of the buffer added to tube 0, and both tubes are shaken. n the bottom layers are transferred, from tube 1 ube 2, and from 0 to tube 1, and the separation inued in this way. It is not necessary to rinse pipette between the transfers. A short swirling he tubes facilitates the separation of the phases. operating time is about 25 min. for a 5-tube es and three hours for 12 tubes.

## APPLICATION

The procedure is exemplified as follows: The dimethyl esters of protoporphyrin rinstein, 1947), mesoporphyrin (Granick, 8, esterification with MeOH—HCl) and matoporphyrin (Nencki & Zaleski, 1900, erification with dizaomethane) were chro- tographed on alumina and crystallized .p. 227°, 216°, and 212° respectively). eighed amounts were saponified together room temperature with 7 M HCl. An quot, diluted with water to the desired centration of the acid, was taken as the eous phase for distribution in a series of es (Fig. 1). Hydrochloric acid of exactly same strength and ether, saturated with h other, were used as the two phases.

To avoid the inconveniences involved in

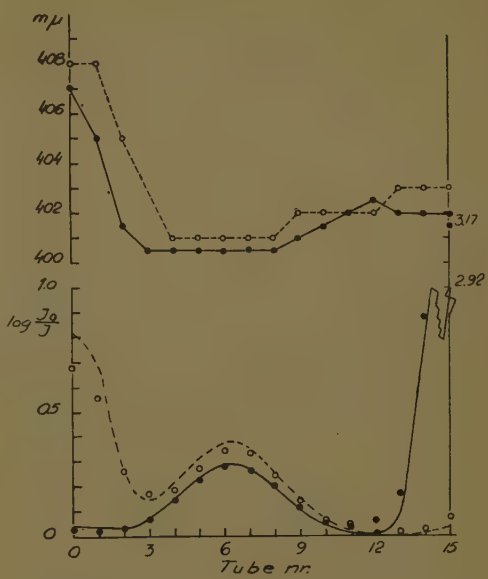


Fig. 1. Counter-current distribution of a mixture of proto-(38.0 μg), meso- (36.4 μg), and hemato- (42.0 μg) porphyrins between ether and 0.12 M HCl. Volume of each phase 8 ml. The ether was subsequently extracted with 7 ml. 1.6 M HCl.
Upper pair of curves:
o - - - o wave-length of top of Soret band in 1.6 M HCl
●—● — — — — 0.12 —

Lower pair of curves:
— — — Calc. light absorption of fractions in 1.6 M HCl
o Found — — — — —
——— Calc. — — — 0.12 —
● Found — — — — —

spectrophotometry of ether solutions, the acid phases were quantitatively removed, and the ether residues then extracted with 1.6 M HCl. The two series of porphyrin solutions in 0.12 and 1.6 M HCl were assayed. All

solutions were read in one sequence with the same instrument (Beckman DU spectrophotometer) and cuvettes, and the top of the Soret band was always approached from the same side. In separate determinations it was found that the saturation of an acid porphyrin solution with ether shifted the wavelength of the Soret band about 1 m$\mu$ towards blue but left the height of the band unchanged, when due correction was made for the dilution by the ether. pH was not influenced by the saturation with ether (glass electrode). The plot of the maximal light absorption in the Soret band region instead of the light absorption at a constant wavelength will, of course, apparently increase the resolving power of the method.

The numerical values of the distribution coefficient K (fraction in ether, subsequently determined in 1.6 M HCl/fraction in 0.12 M HCl) were calculated from pairs of adjacent tubes (Table I) according to the formula (Williamson & Craig, 1947)

$$T_{n,r} = \frac{n!}{r!\,(n-r)!} \times \left(\frac{K}{K+1}\right)^n \times \left(\frac{1}{K}\right)^r; \qquad (1)$$

where r = the number of the tube in question, the first tube in the series being r = 0.
n = (total number of tubes) — 1.
$T_{n,r}$ = sum of both fractions in tube r.
K = distribution coefficient. (Upper phase moving to the left.)

It is also possible to calculate K from the ratio of the fractions in one single tube (Table I). As seen, the two methods give rather divergent results. In the cases of proto- and hematoporphyrin it is obvious

Table I. *The distribution coefficients proto-, meso-, and hematoporphyrins in t system ether/0.12 M HCl.*

| Substance | K-value determined from: ratio of fractions in one tube | | two adjacent tu (formula 1) | |
|---|---|---|---|---|
| | Tube | K | Tubes | K |
| Protoporphyrin . | 0 | 16.39 | 0+1 | 17.86 |
| | 1 | 13.51 | | |
| Mesoporphyrin . | 4 | 1.307 | 4+5 | 1.43 |
| | 5 | 1.242 | 6+7 | 1.35 |
| | 6 | 1.282 | | Av. 1.39 |
| | 7 | 1.299 | | |
| | | Av. 1.283 | | |
| Hematoporphyrin | 14 | 0.0244 | 14+15 | 0.01 |
| | 15 | 0.0265 | | |

that the latter method must be charged wi large possible errors. The value $K_{meso}$ 1.396 gives the better agreement betwe experimentally observed and calculat points.

$K_{meso}$ can also be calculated from t formula (Craig *et al.*, 1945)

$$N = n \times \frac{1}{K+1};$$

where N is the number of the tube with t highest content of solute. N is a continuo function, and its value was interpolated fr Fig. 1 as 6.3, which gives $K_{meso} = 1.38$

From the values $K_{proto} = 17.86$, $K_m = 1.396$, and $K_{hemato} = 0.0186$, from t known, total amounts of each porphyrin, a from separately determined values $E^{1\ cm}_{1\ \mu g/ml}$. (Table II) the light absorpti

able II. $E^{1\ cm}_{1\ \mu g/ml}$ *for hemato-, proto-, and mesoporphyrin.* $\lambda$ *means the wave-length the maximal absorption of the Soret band, and* $E^{1\ cm}_{1\ \mu g/ml}$ *the absorption at this wavelength of a solution with 1* $\mu g$ *porphyrin/ml, in 1-cm cuvette.*

| [HCl] in M | 3.44 | 1.72 | 0.90 | 0.66 | 0.33 | 0.104 | 0.071 | 0.038 | 0.022 |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Hematoporfyrin | | | | |
| $\lambda$ | 404 | 403 | 402 | 402 | 402 | 401 | 401 | 401 | 401 |
| $E^{1\ cm}_{1\ \mu g/ml}$ | 0.652 | 0.675 | 0.686 | 0.709 | 0.736 | 0.769 | 0.756 | 0.756 | 0.756 |
| | | | | | Mesoporphyrin | | | | |
| $\lambda$ | 402 | 401 | 400 | 400 | 400 | 400 | 399 | 399 | 399 |
| $E^{1\ cm}_{1\ \mu g/ml}$ | 0.693 | 0.703 | 0.703 | 0.738 | 0.749 | 0.744 | 0.744 | 0.727 | 0.639 |
| | | | | | Protoporphyrin | | | | |
| $\lambda$ | 408 | 406 | 406 | 405 | 405 | 405 | Clearly seen | | |
| $E^{1\ cm}_{1\ \mu g/ml}$ | 0.411 | 0.419 | 0.416 | 0.422 | 0.400 | 0.330 | precipitation | | |

ues to be expected in the two HCl-fracns were calculated according to formula 1. clearly seen there is a deficit in tubes 0 1 1 (calculated as 5.8 $\mu g$ of protoporrin), and an excess in tubes 10—15 4 $\mu g$ hematoporphyrin). Schumm (1927) nd an equilibrium between proto- and natoporphyrin in strong HCl. It can there- e be assumed that the protoporphyrin had n partly converted into hematoporphyrin our experiment, since the esters were onified with 7 M acid, and the solutions in this medium a few days before the periment, given in Fig. 1, was made. wever, it can also be calculated from mula 1 that there should be very little gether about 0.1 per cent of total) hemaorphyrin in tubes 10, 11, and 12. The stance in excess of calculated amounts in se tubes can thus not be hematoporphyrin. as K ~ 0.111, and could be a mono-vinyl-no-hydroxy-ethyl porphyrin.

The upper pair of curves in Fig. 1 gives the wavelength of the maximal absorption of each HCl-fraction from every tube. The three main fractions can be identified by means of the data in Table II.

Paper chromatography would have given only limited separation of these three substances, either the free porphyrins or their methyl esters had been examined. Adsorption chromatography under standardized conditions (Nicholas, 1951) should have been more effective, but can not very conveniently be applied to microquantities. On the other hand, when distribution (24-tube apparatus, ethyl acetate/glycine buffer pH 1.55) was attempted on a crude porphyrin mixture, obtained from a porphyria urine by extraction with ethyl acetate, only limited separation was seen. Only two fractions appeared, each rather symmetric, in spite of the fact that paper chromatography had disclosed the presence of several components

within each fraction. Although counter-current distribution might give an enrichening of a certain component in a few tubes, paper chromatography is obviously superior for the complete separation in such a case.

## DISCUSSION

The porphyrins are ampholytes. In a sufficiently alkaline medium they exist as anions, and give rather diffuse absorption bands in visible light. When pH is lowered, they become uncharged and insoluble in water but easily soluble in certain organic solvents, where they give four-banded spectra. The transformation from anionic to uncharged form reflects in the behavior in paper chromatography with a system like lutidine-water. At still lower pH the porphyrins acquire two (cf. below) protons and become water-soluble cations. In this form they can be considered as a kind of metalloporphyrins with the two protons corresponding to the metal. Simultaneously their spectra change and become two-banded, like a metalloporphyrin spectrum. At still higher acidity the bands migrate towards longer wavelengths and the height of the Soret band decreases.

Conant, Chow & Dietz (1934) titrated various porphyrins and related substances in glacial acetic acid. They found that in porphyrins two groups with equal $pK'_{HAc}$ behaved as relatively strong bases, whereas another group (possibly two groups) were more weakly basic. Zeile & Rau (1937) distributed porphyrins between ether and acid buffers or dilute hydrochloric acid. The plotting of porphyrin in HCl as *per cent* of total porphyrin against log [HCl] gave curves similar to ordinary dissociation curves but steeper and corresponding to the simultaneous uptake of two protons.

Let P = neutral form of a porphyrin, insoluble in water but soluble in organic solvents (four-banded spectrum) and $PH_2^{2+}$ = cationic form in acid medium (two-banded spectrum). Then

$$P + H^+ \underset{}{\overset{k_1}{\rightleftarrows}} PH^+;$$

$$PH^+ + H^+ \underset{}{\overset{k_2}{\rightleftarrows}} PH_2^{2+};$$

If $k_1 = k_2 = k$,

then

$$pH = pk + \frac{1}{2} \log \frac{[P]}{[PH_2^{2+}]};$$

Hematoporphyrin can easily be obtained in pure form, and its hydrochloric number acid is within an acidity range where pH can conveniently be measured. It was therefore used to test formula 3 (Fig. 2). Obviously the change from ether-soluble form (P) to cationic form ($PH_2^{2+}$) follows formula 3, a favourable circumstance for the ability of the procedure to separate the porphyrins.

It will be noted that in spite of the presence of form P, a hematoporphyrin solution at pH 2—3 shows only the two-banded spectrum. The height of the bands decreases when pH is increased from 1 to 3. The explanation may be that the uncharged particles polymerize, and in such a way that the molecular weights of the aggregates is lower the more acid the solution is. Attempts were made to remove by filtration these aggregates. To a series of glycine or citrate buffers between pH 1 and 4, were added aliquots of hematoporphyrin in 0.1 M HCl. The pH value

e measured, and the tubes left for three
s with occasional shaking to equilibrate.
y were then filtered (Pyrex ultra-fine
s filter), and the filtrates acidified to
M HCl. The result is given in Fig. 2
polymerization of metalloporphyrins in
line media has been described (Shack &
rk, 1947). A further support for the
umption that the porphyrins polymerize
een in the fact that nearly the same in-
ase in light absorption is obtained when
(unstable) porphyrin solution at pH 7.1
cidified to pH 1 or a protein solution is
d (Paul & Avi-Dor, unpublished).

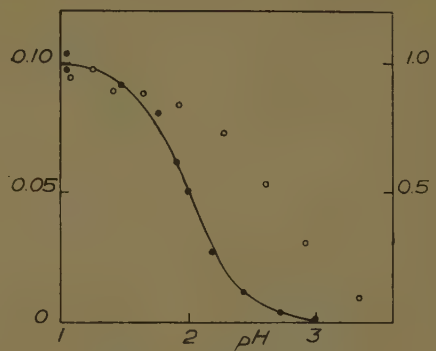


Fig. 2. ● Partition of hematoporphyrin between ether and HCl (right scale: Porphyrin in HCl/total porphyrin in the tube). o Light absorption of filtrate after acidification (left scale). Full drawn curve calculated according to formula (3), assuming pk = 2.00.

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# A MICRO BIURET METHOD FOR PROTEIN DETERMINATIO

## DETERMINATION OF TOTAL PROTEIN IN CEREBROSPINAL FLUID

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Studies on the protein components in biological fluids often require determination of small amounts of protein. The quantitative methods available for this purpose are usually time-consuming or their sensitivity is not satisfactory.

One of the most convenient methods for the determination of proteins is the biuret method. Of the numerous modifications of this principle none of them are worked out for micro estimation, *i. e.* estimation of about 0.1 mg of protein. The reason is, at least in part, that with the photometers commonly used the readings were limited to the visible part of the spectrum. Biuret has an absorption maximum at 560 m$\mu$ and in all the biuret methods the readings are made in this region. Minimum absorption for the biuret color is at 450 m$\mu$ and with shorter wavelengths the absorption increases again as shown by Mehl (1945). In the region of 400—320 m$\mu$ the increase is moderate, thereafter the absorption increases to infinity. Readings of the biuret color at 330 m$\mu$ follow the Lambert-Beers law and as the light absorption at this wavelength is far stronger than at 560 m$\mu$, small amounts of biuret can be distinguished.

The method now to be described differs from other biuret methods only in the composition of the reagents, amount of prote to be determined and choice of waveleng for reading. The method is here applied determination of total protein in cereb spinal fluid, but can also be used for oth protein determinations. A micro procedu is described for quantities of 0.1 to 2.0 r protein and an ultramicro procedure f quantities of 10 to 400 micrograms.

### MICROMETHOD

(0.1—2.0 mg protein)

*Reagents and apparatus*

1. 3 per cent solution of sodium hydroxide.
2. 11 per cent solution of trichloracetic acid.
3. Benedict's reagent.

   Dissolve in a 1 litre beaker 173 g sodi citrate (purum) and 100 g sodium carbon (sicca) by heating, boiling must be avoi Filter the solution warm.

   Dissolve in another beaker 17.3 g cu sulphate ($CuSO_4 \cdot 5H_2O$) in about 100 m distilled water by heating. Mix the two s tions in a 1 litre volumetric flask, cool and to the mark with distilled water. Keep the s tion in a dark bottle with rubber stopper.
4. Photometer. In this investigation a Beck spectrophotometer model B and 1 cm c cuvettes are used. Other spectrophotome which permit readings at 330 m$\mu$ might be used.

### *Procedure*

The total protein content of cerebrospinal fluid cannot be determined directly without previous precipitation because some of the non-protein nitrogen compounds also give a biuret color. The precipitation is carried out in a final concentration of 8.8 per cent trichloracetic acid according to Izikowitz (1941).

Mix in a conical centrifuge tube 1 ml of cerebrospinal fluid with 4 ml of 11 per cent trichloracetic acid. Let stand for about ten minutes at room temperature and centrifuge for 15 minutes at 3000 r/min. Discharge the supernatant fluid by inverting the tube slowly and let the tube drain off for some minutes on a filter paper. Dissolve the precipitate in 4 ml of 3 per cent sodium hydroxide, add 0.2 ml of Benedict's reagent and mix. Prepare a blank by mixing 4 ml of 3 per cent sodium hydroxide with 0.2 ml of Benedict's reagent.. Read the optical density against the blank after 15 minutes in a Beckman spectrophotometer model B at 330 mμ and 1 cm corex cuvettes.

The result in mg per cent is read directly from a standard curve or is calculated by means of the equation:

$$P = 345\ D - 7$$

where P is mg per cent protein and D the optical density.

### *Standard curve*

To get a standard curve the nitrogen content of six sera was determined by the microKjeldahl method and the usual factor of 6.25 was used to convert the nitrogen to protein. Corrections were made for the non-protein nitrogen.

Dilutions 1 : 100 to 1 : 500 of the sera in 0.9 per cent sodium chloride were analyzed and the readings were plotted in a diagram against the protein content. (Fig. 1.)

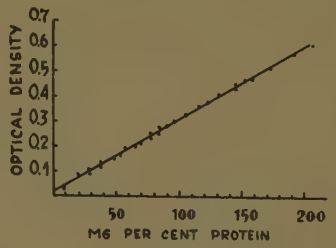



Fig. 1. Standard curve. Readings at 330 mμ and 1 cm corex cuvettes. 30 values obtained from dilutions of six different sera are plotted.

The correlation between the nitrogen content and the biuret color was linear and independent of the alb./glob. ratio. The latter was determined by paper electrophoresis (Goa 1951) and varied from 0.11 to 1.23.

The standard curve was calculated by the method of least squares. The equation of the curve is:

$$D_{330\ m\mu}^{1\ cm} = 0.02 + 0.0029\ P$$

where $D_{330\ m\mu}^{1\ cm}$ the optical density at 330 mμ and a light path of 1 cm and P is mg per cent protein in the sample. In order to calculate the protein content from the optical density the equation can be written:

$$P = 3.45\ D - 0.07$$

Where P is mg protein in the sample.

### ULTRA MICROMETHOD

(10 to 400 micrograms protein)

With the method described, amounts of protein from 0.1 to 2.0 mg can be determined. If it is necessary to determine smaller amounts the sensitivity of the method can be increased by using microcuvettes and carrying out the reaction in a volume of 0.5 ml. In this way amounts of protein from 10 to 400 micrograms can be determined.

*Reagents and apparatus*

1. 26 per cent trichloracetic acid.
2. Microcuvettes of the dimensions 2.5 × 10 × 25 mm. Otherwise as for the micromethod.

*Procedure*

When the protein in very diluted solutions, as in cerebrospinal fluid, is to be determined, precipitate the protein by half the sample volume of 26 per cent trichloracetic acid in small conical centrifuge tubes containing about 2 ml. For example: 0.2 ml of cerebrospinal fluid and 0.1 ml of 26 per cent trichloracetic acid. Centrifuge for 15 minutes at 2500 r/min., then let the supernatant fluid drain off by inverting the tubes. Droplets on the wall of the centrifuge tubes are removed by means of a capillary pipette and suction. Dissolve the precipitate in 0.5 ml of a mixture of 4 ml 3 per cent sodium hydroxide and 0.2 ml of Benedict's reagent. After 15 minutes the solutions are transferred into the micro-cuvettes using a capillary pipette and read at 330 mμ against the mixture of sodium hydroxide and Benedict's reagent as a blank. The cuvettes must be carefully centered.

A standard curve was performed as for the micromethod. The equation for the standard curve of the ultra micromethod is:

$$D = 0.03 + 2.19\,P$$

where D is the optical density and P is mg of protein in the sample.

EXPERIMENTAL

*Absorption spectra. Selection of wavelength*

Determinations of the absorbtion spectra of biuret, protein and reagents are all carried out in a volume of 4.2 ml and read against water. A Beckman spectrophotometer model

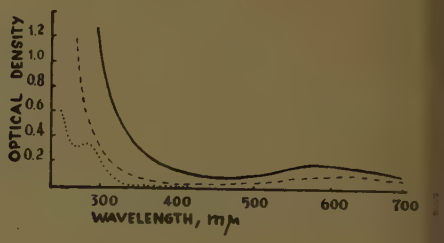
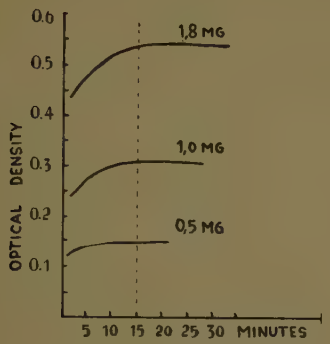



Fig. 2. Absorbtion spectra of the biuret color, t reagents and protein, all in equivalent amoun Biuret ———, reagents — — —, protein .....

DU and ultraviolet accesories were used a in the region of 320 to 700 mμ the readin were also carried out with Beckman spectr photometer model B. The solution of biur consists of 4 ml of 3 per cent sodiu hydroxide, 1 mg of precipitated protein a 0.2 ml of Benedict's reagent. The solutio for the absorbtion spectrum of the reagen consists of 4 ml of 3 per cent sodiu hydroxide solution and 0.2 ml of Benedic reagent. The solution for the protein absor tion spectrum is the same as that for t biuret but with 0.2 ml of water instead Benedict's reagent.

Fig. 2 shows the absorbtion spectra. T biuret color has an absorbtion maximum 560 mμ within the visible part of the spe trum but a far stronger light absorbtion the region of 330 mμ and lower wavelengt The absorbtion of the copper-NaOH-mi ture has a maximum at 630 mμ, for low wavelengths it follows the biuret absorbti but is lower. The absorbtion of the prote is negligible for wavelengths higher th 320 mμ, increasing at lower wavelengths a reaching a maximum at 280—290 mμ. T wavelength 330 mμ has therefore been sele ted as most convenient for reading the biu color. Fig. 2.




. 3. Color development as a function of time. e vertical dotted line indicates 15 minutes, when — as seen — the color is fully developed.

*velopment and stability of the biuret color*

Previous investigations on this problem ve all been concerned with the visible part he spectrum. Robinson & Hogden (1940) ve found the density values at 560 mμ, asured at various time intervals up to 48 irs, to be nearly constant. Measured at ) mμ the density value decreases. This is agreement with the measurements of serer (1936) and Sizer (1937). These hors suggest that the density at 560 mμ ich is stable, is due to the biuret color and t the decrease in density at 700 mμ is to loss of cupric hydroxide from the tem.

Hinsberg & Gleiss (1950) developed the or in a water bath at 37° C during 30 nutes and Lehmann (1944) got a nearly ble color after 10 minutes at room tem- taure. The biuret color as subject to perature variations has been investigated Josephson & Andurén (1949). Using ir method they found a small increase of the biuret color with increasing temperature, but that the ordinary variations in room temperature are of negligible influence.

Fig. 3 shows some experiments carried out in order to follow the color development. The procedure is the same as that for the determination of total protein. As seen, the color is practically fully developed in 15 minutes at room temperature and a slow increase in density follows during the first hour, more marked for high protein content than for low.

Turbidity often develops in the samples when standing. To avoid this error the author has tried different reagents and varied the alkalinity of the solutions. The turbidity is not caused by the copper reagent as it develops even in pure protein-sodium hydroxide solutions. With decreasing alkalinity the development of the turbidity is delayed and with 3 per cent sodium hydroxide as in the described method the solutions remain clear for several hours. The nature of the turbidity is not known. It is not a protein, it contains phosphorus and it is soluble in alcohol. Possibly it is a phospholipid liberated from the lipoproteins as assumed by Lewin & Brauer (1951).

*Reproducibility of the method*

Twelve determinations on two cerebrospinal fluids, one with low and one with high total protein content were carried out. The first series gave an average of 38 mg per cent, lowest value 37 mg per cent, highest value 40 mg per cent and a standard deviation of ± 1.3 mg per cent protein. The second series gave an average of 84 mg per cent, lowest value 82 mg per cent, highest

value 86 mg per cent and a standard deviation of ± 1.5 mg per cent protein. As seen, the standard deviation of estimate is of the same order of magnitude for low as well as for high protein content.

## SUMMARY

A rapid and convenient colorimetric method for the determination of small amounts of protein, based on the biuret reaction and readings at 330 mμ, is described. The "micromethod" allows the determination of 10 to 200 mg per cent protein with an acurassy of ± 1.5 mg per cent. An "ultra micro" modification allows determinations of 10 to 400 micrograms protein.

The method is applied for the determination of total protein in 1 ml of cerebrospinal fluid.

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# OBSTRUCTION OF THE COMMON BILE DUCT N EXPERIMENTAL RENAL HYPERTENSION IN DOGS[1]

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The object of the present work has been examine in dogs how a severe liver injury used by obstruction of the common bile t) influences an experimental renal hyper- sion produced in advance.

Chauffard (Charcot, Bouchard & Bris- d, 1892) was the first to point out, that ric patients in most cases have a low od pressue. About 1900 Gilbert & Gar- (Loeper, 1937) stressed the low blood ssure as a characteristic feature in patients n cirrhosis of the liver; Loeper (1937) Spatt & Rosenblatt (1949) have made same observation. In 1930, Geill found kingly low blood pressure values in acute, ctious hepatitis. Meakins (1932) was first to show, that the blood pressure in ents with arterial hypertension may show ronounced fall when they develop acute, ctious hepatitis, and that this low blood ssure may persist for a prolonged period r the jaundice has subsided. One of us le the same observation in a few patients n subchronic atrophy of the liver (Raa- ou, 1949). In normotensive patients, too, there seems to be a tendency to a lower blood pressure when they are attacked by acute hepatitis, a finding made by Selander (1939) in a large series of patients with hepatitis.

According to investigations in recent years it seems as if the liver may also be of great importance to the substances which are supposed to influence the pathogenesis of experimental, renal hypertension in animals (Braun-Menendez, Fasciolo, Leloir, Munoz & Taquini, 1946). It may thus be mentioned that hypertensinogen in all probability is produced in the liver (Leloir et al. 1942; Page, McSwain, Knapp & Andrus, 1941), as the content of hypertensinogen in the blood in normal and hypertensive dogs is reduced or disappears completely after hepatectomy or injury to the liver (carbon tetrachloride and ethyl alcohol). It may further be mentioned that Haynes & Dexter 1943, 1945) found that the content of hypertensinogen was reduced in human plasma in 6 out of 9 patients with liver insufficiency. With regard to renin, it is the general view that it is destroyed, *inter alia*, in the liver; hepatectomy will thus delay its removal from the blood (Leloir et al. 1942). With regard to the hypertensin-splitting enzyme, hypertensinase, this substance is said also to be present in liver tissue as

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well as in many other parts of the organism; however, its concentration in the blood is said not to be changed in liver insufficiency (Haynes & Dexter, 1945).

Shorr, Zweifach & Furchgott (1945), put forward another theory about the occurrence of two antagonistic substances: the vaso-excitor material (VEM) and the vaso-depressor material (VDM), the function of which should be to regulate the peripheral blood flow and the blood pressure. VDM is produced in the liver, the spleen and the skeletal muscles in the case of tissue anoxia, and it is destroyed in the liver under aerobic conditions. The authors found increased quantities of VDM in the blood and the liver tissue in rats with experimental cirrhosis of the liver; increased proportions of VDM are also said to be found in the blood of patients with cirrhosis of the liver (Schorr, 1947).

Lastly, it can be mentioned that recently Davis, Tanturi & Tarkington (1949) and Davis & Tanturi (1951), have published some experiments with Goldblatt dogs which, both with regard to the procedure and the results of experiments, closely resemble the experiments on dogs to be reported here; in Goldblatt dogs they have undertaken partial clamping of the portal vein and the hepatic artery, either separately or simultaneously. When the flow of blood through the liver had been sufficiently reduced (which manifested itself, *inter alia*, by the appearance of fatty infiltration in the liver tissue), they observed that the blood pressure fell.

Our interest in the question of the importance of liver function to the pathogenesis of human essential hypertension originates from the great hepatitis epidemic in Denmark during World War II; numerous patients died under the pathological pict of subchronic liver atrophy (Bjørneboe Raaschou, 1949). With very few exceptio the patients who died were women over t age of 45. In this large group of women, w were all in the age-groups where the f quency of essential hypertension is norma high, it was striking to observe that hyp tension occurred with a very low frequen (Raaschou, 1949). In a few patients w had been suffering from hypertension for prolonged period this disorder subsided wh they developed subchronic atrophy of t liver.

The strikingly low frequency of arter hypertension in subchronic atrophy of t liver and in cirrhosis of the liver, and t fact (Bradley, 1948) that the function of t liver is found normal in essential hyp tension in man, form the basis of the f lowing hypothesis, on which our exper mental work is based: a normal or or slightly damaged liver function is a necessa condition for the development of hyperte sion in man, whereas a severely damag liver function will prevent the developme of hypertension, or will obviate an alrea existing hypertension.

## METHODS

a) *Measurement of the blood pressu* The mean blood pressure was daily det mined in the femoral arteries by means an ordinary mercury manometer .

b) *Operation to produce renal hyperte sion.* Goldblatt's technique with bilate constriction of the renal arteries by mea of silver clamps was used (Goldblatt, Ly Hanzal & Summerville, 1934; Goldbl

40). In most of the dogs the procedure s as follows: silver clamps were first plied to both renal arteries, which were strictedto about half the normal lumen. the blood pressure did not rise sufficiently, unilateral reoperation was made two or ee weeks later in order to increase the con- iction a little. It is difficult to obtain a finite, intended degree of constriction of renal arteries and a resulting, sufficient gree of hypertension: in 15 out of 37 ldblatt dogs we inadvertently applied too eat a constriction, which led to uremia and ath, in 11 dogs earlier than the 9th day er the operation. In 20 out of 37 dogs the pertension was successful, in 6 after one eration, in 13 after reoperation, whilst one g had to be operated on 3 times in all; in o dogs this technique did not cause hyper- sion. In a few of the dogs we chose a ferent procedure in order to avoid the velopment of a collateral circulation from renal capsule to cortex after constriction the renal arteries; in some cases we had served such a collateral circulation at the operations, and this may possibly contri- te to a slowly progressing fall of the vated blood pressure which was undesir- le, as we wanted to study the possible effect a subsequent bile duct-operation on a *stant* high blood pressure. Therefore, we nbined a slight, bilateral constriction of renal arteries with enclosure of both dneys in polyethylene bags which were htly closed around the stalk of the kidney. is method has been satisfactory.

c) *Operation to produce complete obstruc- n of the common bile duct.* In the first ee dogs (Dog Nos. 1, 2 and 3) a single ligature was placed round the middle of the common bile duct. This interference did not cause any permanent complete obstruction, for after a rapid and great increase of the serum bilirubin concentration lasting 7, 9 and 13 days respectively a gradual fall of the concentration towards normal values was observed in the course of 1 to 2 months. At the autopsy it was found in all three dogs that the passage of bile had been re-established in the common bile duct, as the ligature had cut through part of the wall; consequently the biliary stasis in the liver had been obviated to some extent.

The method has been changed as follows: a silk ligature was placed round the distal part of the isolated common bile duct (considering the fact that the hepatic duct in dogs often opens into the lower part of the common bile duct) and proximal to this a silver clamp (the same type as used for the renal arteries) was applied, so that it produced complete obstruction. In addition, an excision of the common bile duct was made to an extent of 2 to 3 cm distal to the ligature in most of the dogs. As a rule, this interference causes higher serum bilirubin concentrations persisting for a longer period. In this manner 12 operations have been made; in 7 dogs the operation was successful, in one of these *(Dog No. 9, Sokrates)* the obstruction was, however, intermittent (this dog had had no resection); in 4 dogs perforation of the common bile duct soon occurred, resulting in bile peritonitis and death; unfortunately we lost one dog because of aspiration after the operation.

d) *Postoperative treatment.* 1) After Goldblatt operations the dogs were daily given 1 litre of 10 per cent glucose by mouth or subcutaneously until their ingestion of

Table I. *Obstruction (with a single silk ligature) of the common bile duct in three hypertensive dogs; the obstruction was only complete for 7, 9 and 13 days.*

| Dog No. | Name | Normal mean blood pressure level (mm Hg) | Kidney operation | Complete obstruction of the common bile duct. (days) | High blood pressure level (mm Hg) | Blood pressur fall down to (mm Hg) |
|---|---|---|---|---|---|---|
| 1. | Bill | 100—110 | Constriction of both renal arteries | 9 | 160—170 | 120—130 |
| 2. | Ulf | 120 | Constriction of both renal arteries | 7 | 160 | 125—130 |
| 3. | Treu | 115—120 | Constriction of both renal arteries | 13 | 165 | 130—140 |

food had become normal. 2) Because of the retention of bile the dogs will, as a rule, lose some appetite; after the obstruction of the common bile duct the dogs were therefore daily given 200 ml of a 50 per cent glucose solution and 300 ml of an emulsion of amino acid (800 g of a 15 per cent aminolin solution (»*Orthana*«, an acid-hydrolyzed casein preparation)/2 litres), starch (800 g amylum triticum/2 litres), peanut oil (200 g oleum arachidis/2 litres), NaCl (6 g/2 litres) and KCl (4 g/2 litres) by stomach tube, and vitamins A, B, D and K parenterally. Thus the dogs during the period of obstruction of the bile duct were given sufficient calories and amino acids to cover the nitrogen requirements.

e) *Laboratory examinations.* In order to follow the biochemical changes in the blood, the following analyses of the plasma were made: 1) Urea (Van Slyke & Kugel), 2) bilirubin (With's modification of Jendrassik & Grof), 3) alkaline phosphatases (Buch) and 4) fractionated plasma protein determination (Kjeldahl-Howe). Biopsy materia was removed from the liver and kidneys i the course of the operations and *post morte*

## RESULTS OF EXPERIMENTS

The results of the experiments will b dealt with in three different groups:

I. Obstruction of the common bile duct i hypertensive dogs.
II. Sham operation on the common bil duct in a hypertensive dog.
III. Obstruction of the common bile duct i a normotensive dog.

I. In the first three dogs (Dog. Nos. 1 and 3) — as mentioned above — the passag of bile was re-established in a short tim If we only consider the blood pressure durin the period when the obstruction of the com mon bile duct — judged from the serum bilirubin concentrations — was complete they all displayed an unquestionable fall o the blood pressure (Table I). The genera health of the dogs was good during th period, and it would therefore seem justif

Table II. *Complete obstruction of the common bile duct (ligature, silver clamp, resection) in 6 hypertensive dogs. Resection of the common bile duct was not performed in dog No. 9.*

| Dog No. | Name | Normal mean blood pressure level (mm Hg) | Kidney operation | Number of days from the Goldblatt operation to the bile duct operation | High blood pressure level before the bile duct operation (mm Hg) | Blood pressure fall to (after the bile duct operation) (mm Hg) | Good general health (days after the bile duct operation) | Ascites |
|---|---|---|---|---|---|---|---|---|
| 4. | Jeanne d'Arc | 117 | Right nephrectomy<br>Constriction of the left renal artery.. | 40 | 170—195 | 110—130 | 51 | + |
| 5. | Türck | 100 | Constriction of both renal arteries | 35 | | | | |
| | | | Reoperation on left side ..... | 22 | 180—200 | 130 | 28 | ÷ |
| 6. | Carmen | 90—100 | Constriction of both renal arteries | 25 | 150—180 | 105 | 10 | + |
| 7. | Don Ranudo | 80—100 | Constriction of both renal arteries | 47 | | | | |
| | | | Reoperation on left side ..... | 35 | 165—185 | 135—150 | 17 | + |
| 8. | Odysseus | 80—100 | Constriction of both renal arteries | 126 | | | | |
| | | | Reoperation on left side ..... | 80 | 170—200 aver: 185 | 140—145 | 10 | ÷ |
| | | | Left nephrectomy. | 49 | | | | |
| 9. | Sokrates | 116 | Constriction of left renal artery ..... | 161 | | | | |
| | | | Constriction of right renal artery | 141 | | | | |
| | | | Sham operation on the common bile duct..... | 37 | 185 | 110—130 | 138 | + |

e to attribute a certain importance to this od pressure fall.

We shall account in greater detail for the uence exerted by complete constriction the common bile duct for a longer time experimental hypertension in 5 dogs able II). With the above mentioned sur- al technique it was possible to produce a sufficiently long-standing obstruction of the bile duct during which the general health was sufficiently good for a prolonged period for an estimate of the blood pressure to be made. Mention may be made first of a dog with hypertension of short duration on which obstruction of the bile duct was performed and which then developed biliary cirrhosis.

bilirubin concentr. of the blood mg%
alkaline phosphatase units
mean blood pressure
blood urea mg%
Jeanne d'Arc
Autopsy
Delivery
liver biopsy (laparotomy)
Cholecysto-duodenostomia
Occlusion and resection of the common bile duct
Clamping of the left renal artery
Ascites +
July August Septbr Octbr Novbr Decbr Jan Febr March April May June Aug - Septbr

Fig. 1. Dog No. 4, female. Right nephrectomy was performed on May 19, 1950. July 26, left main renal artery was moderately constricted. September 4 obstruction and resection of the common bile duct. October 25 anastomosis between the gall-bladder and duodenum. December 20 laparotomy with liver biopsy. June 27, 1951 delivery. September 11 autopsy.

*Dog. No. 4* (Jeanne d'Arc) (Fig. 1). On May 1950, a right nephrectomy was performed. Normean blood pressure averaged 117 mm Hg. July 26 a silver clamp was applied to the left l artery, which was constricted to about half normal lumen. During the next 40 days the d pressure rose to values between 170 and mm Hg. On September 4 a silver clamp and a ligature were applied to the common bile duct, ch was completely constricted; 2 cm of the was excised distal to the clamp. Histological mination of the liver showed normal liver ue. After the operation on the bile duct the m bilirubin and the alkaline phosphatases soon to 14—16 mg per cent and 500 units respecly. These values later showed a slight gradual (in spite of persisting complete obstruction of bile duct). This period with obstructive jaundice, ng which the stools were constantly clayred, lasted 51 days. Soon after the obstruction been established, an incipient fall of the mean d pressure was observed, and in the course of ut 30 days it fell to the original level. The dog lively and well all the time, ate and drank with l appetite. Towards the end of this period tes was first ascertained. On October 25 an stomosis was made between the large and ended gall bladder and the duodenum; there about 1 litre of icteric ascites fluid in the toneum. The liver was yellowish brown, with nounced signs of biliary stasis. Histological mination of the liver showed: enlargement of periportal spaces, moderate proliferation of the ducts and incipient cirrhosis; further, a modecentro-acinose jaundice of the obstructive type. dog recovered soon after the anastomosis had established and was in the best of health; he course of a week the serum bilirubin conration fell to normal values, and remained nal for the rest of the time; the alkaline phosases, which also showed a fall after the anastois had been made, did not fall to normal values were moderately increased (from 20—90 units) ng the rest of the experimental period. As the es persisted and as a low serum albumin perage and a positive Takata-Ara reaction were rtained, there was reason to suppose that the had developed a permanent disorder of the liver. The stools were brown every day, which in connection with the normal serum bilirubin concentration indicated a normal passage of bile to the intestine. During this period the blood pressure remained unchanged at normal values — about 120 mm Hg. On December 20, 1950, a laparotomy with biopsy of the liver was made; the liver was of a deep green color, firm, tough and with sharp edges; there was ascites, and numerous adhesions with copious bleeding were found between the omentum and the anterior abdominal wall. Histological examination of the liver biopsy showed unchanged acinose structure and considerably enlarged periportal spaces with pronounced proliferation of the bile ducts. The periportal spaces further showed a pronounced subchronic, simple inflammatory infiltration and moderate cirrhosis. No signs of occlusions of the bile ducts.

The dog was observed for a total of 372 days after the obstruction of the common bile duct had been established. Its general health remained quite uninfluenced, the blood pressure remained unchanged at the normal level, there were varying amounts of ascites, moderately increased phosphatase values and a low serum albumin level (serum albumin per cent was 3.34 before the obstruction of the bile duct, 1.33—2.44 in the period after). In the spring of 1951 the dog became pregnant and after an apparently quite normal pregnancy it was delivered of 7 puppies on June 27, 1951. For the next two months no examinations were therefore made. When the examinations were resumed from August 21 to September 11, conditions wer found unchanged: normal blood pressure, ascites and strongly positive Takata-Ara reaction. During the entire experiment the weight had increased by 5 kilos. On September 11 the dog was killed under nembutal anaesthesia. *Autopsy* showed about 0.25 litre of ascites. The liver was reduced in size, firm, nodular. The color was greyish yellow, the anterior edge sharp. The density was markedly increased everywhere; it was thus impossible to press a finger through the tissue. Macroscopically it suggested quite an advanced biliary cirrhosis. Between gall bladder and duodenum there was an anastomosis as thick as a pencil, which must have functioned normally. The bile ducts were not distended. Histological examination of the

liver: advanced biliary cirrhosis and diffuse steatosis, no jaundice.

Mention will now briefly be made of 4 dogs *(No. 5 (Türck), No. 6 (Carmen), No. 7 (Don Ranudo)* and *No. 8 (Odysseus))*, 3 with brief and one with more prolonged arterial hypertension, in which the obstruction of the bile duct was followed by a marked fall of the blood pressure. Table II shows: a) the normal preoperative mean blood pressure levels, b) the kidney operations, c) the number of days from the Goldblatt operation to the bile duct operation, d) the high blood pressure levels before the bile duct operation e) the blood pressure levels after the bile duct operation, f) the number of days after the bile duct operation during which the general health was good, and g) the cases in which ascites occurred after the bile duct operation.

II. As it was possible that the surgical interference proper and the anaesthesia might be responsible for the fall of the blood pressure observed, the effect on the blood pressure of a sham operation on the common bile duct was examined in the next animal, which had first developed a long-standing arterial hypertension. Later the effect of prolonged obstruction of the bile duct was examined. The sham operation took the form of a laparotomy, with all the usual observances, at which the common bile duct was exposed in its entire length. The operation was then concluded without any attempt at obstruction of the common bile duct. Our intention was that the operation should be of the same duration as the other operations on the bile duct.

*Dog. No. 9* (Sokrates) (Fig. 2). (Table II.) Normal mean blood pressure: 116 mm Hg. On January 5, 1951, a silver clamp was applied to the left re artery, which was constricted to about one-fifth the normal lumen. During the next 20 days the blo pressure remained unchanged. Immediately af the operation the blood urea rose for a short ti to 112 mg per cent, and then fell to normal valu On January 25 a silver clamp was applied to t right renal artery, which was only very sligh constricted. In the course of the next month arterial hypertension developed, varying betwe 160 and 200 mm Hg, averaging 170 mm Hg. May 9 a sham operation, as mentioned, was ma on the common bile duct. The dog was observed for 37 days. It was quite well, had a go appetite, and the stools were brown. *To begin wi the blood pressure remained unchanged at the p operative level, about 165 mm Hg; it then rose little — to about 185 mm Hg.* On June 15 a silv clamp and silk ligature were applied to the comm bile duct, but no excision of the duct was ma In the course of the first 4 days after the ope tion the serum bilirubin value rose, and the d passed clay-colored stools. During the next 3 we the stools were brown, and the serum biliru level was normal. From July 15 the stools beca clay-colored again, whilst at the same time serum bilirubin concentration rose abruptly values between 5 and 8 mg per cent. During entire period that followed the stools were mos clay-colored but there were days in between wh they were slightly brownish. The operation thus produced an intermittent obstructive jaund in spite of silver clamp and silk ligature the p sage of bile was reestablished for periods. W regard to the blood pressure, it fell immediat after the operation on the bile duct to 125—130 Hg, then rose to 160 mm Hg, after which simultaneously with the occurrence of perma obstructive jaundice — it showed an even towards the original normal blood pressure of dog. During the whole of this long period dog was quite well and had a good appetite vomited only seldom, and the blood urea remai unchanged at normal values — from 20 to 40 per cent. On September 5 abdominal punc showed copious ascites fluid, whilst at the s time the serum albumin had fallen from 3.32— to 0.99—1.24 per cent. On November 8 the

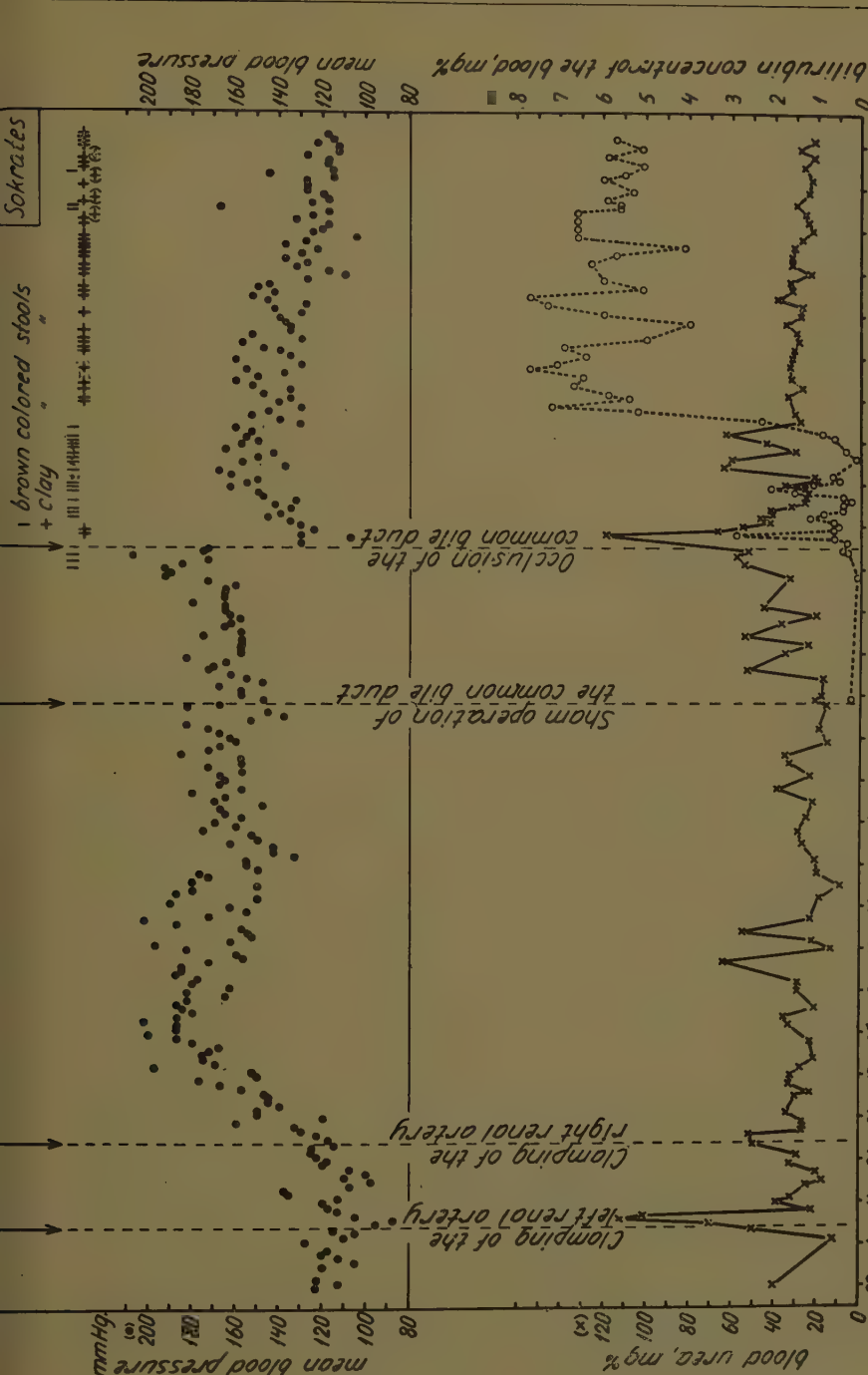
Sokrates
1 brown colored stools
+ clay
Occlusion of the common bile duct
Sham operation of the common bile duct
Clamping of the right renal artery
Clamping of the left renal artery
mean blood pressure mm Hg.
blood urea, mg%
bilirubin concentr. of the blood, mg%
Dec. Jan. Febr. March April May June July Aug. Septbr.

Fig. 2. Dog No. 9, male. January 5 left main renal artery was severely constricted. January 25 right main renal artery was slightly constricted. May 9 sham operation on the common bile duct. June 15 obstruction of the common bile duct (with silver clamp and ligature, but without resection).

died of uremia. At autopsy there were about 4 litres of icteric ascites fluid; there was no bile in the peritoneum and no lesion of the biliary tract. The liver was atrophic, the anterior edge was sharp, the density was increased, and there were pronounced signs of biliary obstruction. The gall-bladder and the hepatic ducts were moderately distended. The common bile duct was much distended proximal to the ligature. The loop of the ligature and the silver clamp were found in the lumen of the common bile duct; the ligature had thus cut through part of the wall. At the site of the ligature there was a small passage for bile to the duodenum.

III. In conclusion it may be mentioned that a complete obstruction of the common bile duct has been made on a normotensive dog *(Dog No. 10, Aida)*. The general health of this dog was good during the first 28 days after the operation; there was no change of the mean blood pressure during this period.

*The plasma proteins after obstruction of the bile duct.*

The changes in the plasma proteins were uniform. In all 6 dogs a fall of the relative albumin percentage (serum albumin per cent/total protein per cent × 100) was observed, whilst at the same time the serum globulin percentage rose. In 5 out of 6 dogs there was a fall of the serum albumin percentage, and in 4 out of 6 dogs a fall of the total protein concentration was observed.

By means of a sufficient supply of amino acids — as mentioned — we made sure that nitrogen inanition did not occur. When these serum protein changes are compared with the fact that 4 out of 6 dogs developed ascites after obstruction of the bile duct, they must be taken as a proof that a complete obstruction of the common bile duct in dogs produces as massive changes of the liver function as we desired.

## DISCUSSION

With regard to the question of explaini what may be the cause of this fall of the blo pressure, we are at present practically in t dark. We shall for the time being confi ourselves to give an outline of three pos bilities.

1. The fall of the blood pressure might unspecific in the sense that it might eith be due to the surgical interference and t anaesthesia, or to the influence on the gene health which the liver injury (in connecti with the already existing renal disorder) m exert. We believe that this can be consider out of the question. The results obtain with dog No. 9, which had both a sha operation and later an obstruction of the co mon bile duct, seem to suggest that the oper tion *per se* does not produce any prolong fall of the blood pressure. In this connecti attention may, however, be drawn to num ous operations (hypophysectomy (Goldbla Braden, Kahn & Hayt, 1942), cutting the ventral spinal routs (Goldblatt & Wa man, 1937), excision of both carotid sinu (Goldblatt, Kahn, Bayless & Simon, 194 and resection of splanchnic nerves (Go blatt, Gross & Hanzal, 1937)) made in hyp tensive dogs, in which no fall of the blo pressure could be registered. Adrenalecto is actually the only surgical interfere which has been capable of producing a f of the blood pressure in Goldblatt d (Goldblatt, 1937). As to the possibility t alterations in the general health caused fall of the blood pressure, it may be stres again that we have reckoned only with s falls as were registered during the peri when the general health of the dogs satisfactory.

The retention of bile in the blood will
bly exert a toxic effect on the circula-
an explanation which might be in
rdance with a number of earlier experi-
s with intravenous injection of total bile
rall & Carlson, 1928; King & Stewart,
; Meltzer & Salant, 1905), bile salts
ersberg & Taubenhaus, 1926; Horall &
son, 1928; Loewit, 1881; Traube, 1864)
biliverdin, (King & Stewart, 1909)
neans of which a fall of the blood pres-
and bradycardia could be produced in
als. Mention may also be made of
er perfusion experiments by Rywosch
8). He showed in frogs and isolated
ns of mammals that an admixture of
l quantities of bile salts to the perfusion
d will produce dilatation of the vessels.
results in dog No. 4, which after a fall
ne blood pressure in the course of ob-
ction of the common bile duct still had
low blood pressure in spite of the fact
the retention of bile was obviated by
nastomosis operation, and which devel-
cirrhosis of the liver, might seem to
est that the fall of the blood pressure
ot at any rate be due only to retention
ile. The results would rather suggest
also an influence on liver function may
ne cause of the fall of the blood pressure.
This third possibility will have to be
unted for in greater detail in future. In
connection it may be imagined either
the obstruction of the bile reduces the
ation in the liver of a substance which is
ssary for the occurrence of hypertension
s has been postulated in the case of
rtensinogen — or that the injury to the
gives rise to an increased production
of a substance, which reduces the contraction of the arterioles — as has been mentioned in the case of vasodepressor material (VDM).

## SUMMARY

The frequency of arterial hypertension in subchronic atrophy of the liver is very low in relation to what might be expected according to the age-distribution of the patients. The hypothesis was established that a severely damaged liver function may prevent the development of arterial hypertension, or may obviate an already existing hypertension, and that a normal or only slightly influenced liver function is a necessary condition for the development of hypertension.

*Summarizing,* it can be said about the results of our experiments that 6 hypertensive dogs (with experimental, renal hypertension according to Goldblatt's method) — 4 having had hypertension of short duration, 2 having had a more prolonged hypertension — displayed a gradual fall of the blood pressure towards or down to the normal, pre-operative values after obstruction of the common bile duct had been established. These blood pressure falls were observed in dogs in good health, which were sufficiently fed.

In one dog biliary cirrhosis with ascites and decreased serum albumin per cent developed after obstructive jaundice for 51 days; the high blood pressure fell in relation to the obstruction of the bile duct and remained at normal values in spite of the fact that the obstruction of the bile duct was later on obviated by cholecystoduodenostomy and in spite of permanent constriction of the left renal artery (this dog had one kidney only).

In one hypertensive dog a sham operation

on the common bile duct did not change the high blood pressure, whereas a subsequent obstruction of the common bile duct resulted in a gradual fall of the blood pressure to normal, pre-operative values.

In one normotensive dog the obstruction of the common bile duct produced no material changes of the blood pressure for 28 days after the operation.

The changes of the serum proteins observed — a fall of the serum albumin and a rise of the serum globulin with positive Takata-Ara test — and the development of ascites in 4 out of 6 dogs suggest that a massive influence is exerted on the liver function after complete obstruction of the common bile duct in dogs.

The cause of the fall of the blood pressure after obstruction of the common bile duct in hypertensive dogs is not known. It is not very probable that it should be unspecific, or caused by retention of bile in the blood. The experiments do not exclude the possibility that changes of the liver function may be of pathogenetic importance. Complete obstruction of the common bile duct produces a fall of the serum albumin per cent and a rise of the serum globulin per cent and, in many cases, it also gives rise to ascites.

Further investigations must aim at examining hypertensinogen and the vasodepressor material (VDM) under these experimental conditions.

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# THE OCCURRENCE OF CHYLOMICRONS IN THE BLOO IN YOUNG AND OLD INDIVIDUALS

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## INTRODUCTION

It is an old-established observation that part of the fats in the blood occur in the form of particles, the so-called chylomicrons. They measure from ½ to 1 $\mu$ and are visible in dark-field illumination; they consist chiefly of neutral triglycerides but also contain cholesterol, and probably phosphatides and globulins as well (Blix, Tiselius & Svensson, 1941, Cunningham & Peters, 1938, Jones, Gofman, Lindgren, Lyon, Graham, Strisower & Nichols, 1951, Swank & Wilmot 1952). In the fasting individual they are present in the blood in a very low proportion, which increases very much after ingestion of fat; the greater part of the postalimentary rise in the content of triglycerides in the blood occurs in the form of chylomicrons. (Swank & Wilmot, 1952). Therefore the presence of chylomicrons has chiefly been studied in connection with investigations of the fat resorption (Gage & Fish, 1924); (Frazer & Stewart , 1936, 1937, 1939). However, in recent years various investigators, especially Moreton (1947, 1948, 1950), have stressed the possible rôle of the chylomicrons in the development of atheromatosis. Moreton bases his theory upon investigations by Huper (1939) who in experiments on rats and rabbits was able to produce vascular lesions correspon ing closely to atheromatosis by the injecti of macromolecular particles, *e. g.* polyvi alcohol, methyl cellulose and the like.

Moreton (1947) found that the admi stration of fat to human subjects produc a rise in the number of chylomicrons, whi increased in size at the same time; this cor sponds exactly to the findings in diabet myxedema, nephrosis and experimen atheromatosis. His theory thus imports t these large and numerous particles of have a special aptitude to penetrate into t intima, after which the neutral fat and t phosphatides are dissolved, while choleste is deposited. Becker, Meyer & Nechel (1949, 1950) have moreover found that t number of chylomicrons is larger in o individuals than in the young, and t especially the rise following the ingestion fat is both higher and of longer duration the old. They thus explain that age a atheromatosis have a bearing upon one a other. Zinn & Grifith (1950) have report that patients with coronary sclerosis and d betics have an increased number of la chylomicrons in the blood. However, no of these authors have been able to prove t rôle which chylomicrons might possibly p in atheromatosis.

ven though the investigations by the man-group (Gofman, Lindgren, Elliot, tz, Hewitt, Strisower, Herring & Lyon, , Gofman, Jones, Lyon, Lindgren, Stris-, Colman & Herring, 1952, Jones et al. ), into lipoproteins by means of ultra-rifugation decidedly contradict the above-tioned theory, we nevertheless considered it would be of interest to examine the rrence of chylomicrons in the blood in ng and old individuals, *inter alia* to have sis of comparison for our examinations he effect of heparin and protamine sul-e on chylomicrons.

## TECHNIQUE

Ve have used the technique described by zer & Stewart (1937) for counting omicrons in the dark field.

he blood is taken from the ear in micro-tubes; these are left at 37° C until a ll drop of serum can be removed with pillary tube and placed on a slide which been thoroughly defatted (see below). drop is covered with a cover-glass which eres to the slide by capillary action, and preparation is placed in the dark field oil immersion; the magnification is ) diameters.

or the purpose of counting the field is ded into smaller squares by means of ometer with netlike division. The num-of chylomicrons is counted in ten small res in two different parts of the pre-tion. The chylomicrons appear in the field as more or less well-defined light s which perform Brownian movements. zer & co-workers (1937) distinguish veen 3 kinds of particles: "Dull", "small ht" and "large bright". However, we considered that we could only distinguish large (*i. e.* large bright), from the total number (*i. e.* dull plus large and small bright).

It must be strongly emphasized that there are several sources of error in chylomicron-counts: —

(1) Impurities of slides and cover-glasses, especially contamination with fat may interfere with the counting; we therefore cleansed them in chromic sulphuric acid for 24 hours, after which they were placed in alcohol and thoroughly wiped in a clean dry cloth.

(2) The drop of serum used must be so small that the cover-glass adheres to the slide by capillary action; this prevents the presence of currents which would render counting impossible.

(3) It should be noted that the count is made in an unknown volume; some investigators have used very thin quartz counting-chambers, which, however, did not increase the accuracy.

(4) When the microscope is adjusted, 3 layers can be seen; an upper layer in which the particles are motionless, as they adhere to the cover-glass, a lower in which they adhere to the slide, and finally an intermediate layer in which the particles are moving about freely; the counting is made in the last-mentioned layer, and it is important to count only the particles which are moving and not to change the focal distance while counting.

(5) Chylomicrons perform Brownian movements, which renders counting difficult and necessitates a certain routine.

The error in duplicate analyses in our material has been 7 per cent.

In repeated countings in the same blood sample it appeared that when it had been left

Table I

| | | In fasting subjects | Time after ingestion of cream | | | | |
|---|---|---|---|---|---|---|---|
| | | | ¾ hour | 1½ hours | 2½ hours | 3½ hours | 5 hours |
| 10 young | Average ... | 16 | 28 | 66 | 68 | 57 | 39 |
| | variation ... | 10–23 | 11–50 | 30–100 | 46–100 | 16–91 | 10–55 |
| 10 old | Average ... | 7 | 15 | 30 | 45 | 44 | 37 |
| | variation ... | 4–14 | 5–41 | 13–43 | 28–66 | 13–80 | 16–70 |

Number of chylomicrons in fasting subjects and for 5 hours after ingestion of 200 ml of a 13 per cent cream.

for 12 hours the number of chylomicrons had not changed.

Countings of chylomicrons were made partly while the subject was fasting, partly at various times after ingestion of 19.5 g of fat, administered in the form of 150 ml of a 13 per cent cream.

## SERIES OF PATIENTS

Our series consisted of 50 patients, divided into 2 groups aged 20—50 years and over 50 years respectively. All the subjects were males; none of them had myxedema, diabetes or nephrosis, and none displayed clinical signs of manifest atheromatosis.

## RESULTS

The total number and the standard deviation while the subject was fasting and at various times after ingestion of cream in 10 "young" and 10 "old" persons are shown in Table I. Figure 1 shows the same values plotted in a curve.

It appears that there is a considerable rise in the number of chylomicrons in the course of resorption of fat.

The curve seems to rise more quickly in the "young" and to fall more slowly in the "old". The curve also seems to show that the maximum is reached a little sooner the "young" than in the "old". However, t 2½-hour value represents the maximal nu ber in both groups.

It appears moreover that the curve repr senting the "young" is at a higher level th the one giving the results in the "old". order to examine whether there is any u questionable difference between the tw groups, considering the great individu standard deviation of the values, the 2½ hour value was examined in a larger seri comprising 25 "young" and 25 "old" ind

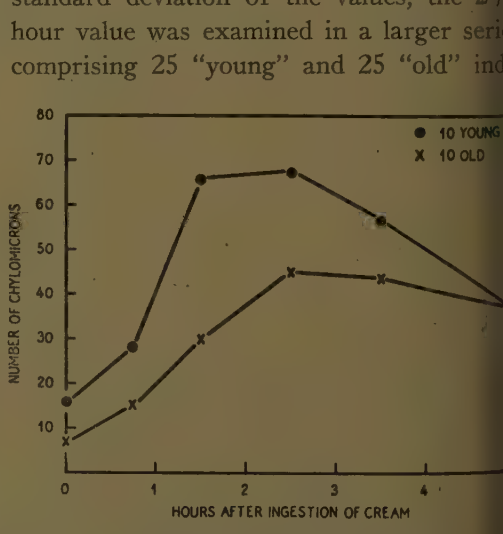


Fig. 1. The total number of chylomicrons in serum of ten young and ten old subjects follow the ingestion of 19.5 g of fat.

Table II

| | In fasting subjects | | 2½ hours after ingestion of cream | |
|---|---|---|---|---|
| | Total | Large | Total | Large |
| ung Average | 12 | 6 | 68 | 43 |
| variation | 6–23 | 3–9 | 41–100 | 21–74 |
| Mean error | | | 18,1 | 14,6 |
| Mean error of mean figure | | | 3,62 | 2,92 |
| d Average | 10 | 4 | 46 | 28 |
| variation | 4–24 | 2–7 | 21–86 | 14–42 |
| Mean error | | | 16,3 | 10,1 |
| Mean error of mean figure | | | 3,25 | 2,02 |

ber of chylomicrons in fasting subjects and hours after ingestion of 200 ml of a 13 per cream.

als). In this examination a distinction also made between "total" and "large" omicrons. Table II gives these results; nean error and the mean error of the n figure. It appears that there is a signi- nt difference between the values in the ng" and the "old", and that both the number and that of the "large" chylo- ons are lower in the "old" than in the ng".[1]

## CONCLUSION

ur examinations thus apparently show when the number of chylomicrons is ted in the course of 5 hours after a fat entration test a slighter rise is found in iduals over 50 years than in those under 50. As already mentioned, Becker & co-workers (1949, 1950) found the reverse, as they state that the values in older individuals are always higher than in the young; thus they found 3 hours after ingestion of fat that the values in the old were twice as high as those found in the young. They administer 0.50 g of fat per kg body weight in the form of oleomargarine, which has probably been a mixture of animal and vegetable fats.

Although our fat load thus does not correspond to the one used by these authors, we would nevertheless consider it improbable that this difference should be capable of producing a fundamental change in the results found in "young" and "old" individuals. Thus our examinations could not confirm the results of the experiments performed by Becker & co-workers.

## SUMMARY

The number of chylomicrons has been counted while the subject was fasting and for 5 hours after ingestion of 20 g of fat in the form of cream in 10 persons under 50 years and 10 over 50. The shape of the curve suggests that the maximum occurs a little earlier in the young than in the old; however, the determination made 2½ hours after the ingestion of fat represents the maximal number in both groups. In old individuals the curve is at a lower level than in the young.

The number of chylomicrons present 2½ hours after the ingestion of fat has been counted in a total of 50 persons, 25 young and 25 old. The values in the young are higher than in the old, and the difference is significant.

[1] The difference between the mean figures for ng" is 22 and for "large" is 15. These differences greater than $3 \times \sqrt{3.62^2 + 3.25^2}$ and $\sqrt{2.92^2 + 2.02^2}$ respectively.

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# THE EFFECT OF HEPARIN AND PROTAMINE SULPHATE ON THE OCCURRENCE OF CHYLOMICRONS IN HUMAN BLOOD

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n 1943 Hahn found that heparin was ble of reducing the postalimentary lipe- in dogs as determined by measurements he opacity of the serum. Weld (1944) confirmed this and shown that it is also case in cats and humans. Waldron & dman (1948) found the same effect of rin-related, sulphonated polysaccharides. lerson & Fawcett (1950) have found heparin reduces the surface tension in m and consider this the cause of the ltaneous decrease of the opacity of the m. The antilipemic effect of heparin is seen *in vivo*; in *in vitro* experiments dmixture of heparin to postalimentarily que serum produces no effect; but plasma n fasting individuals who have been n an intravenous heparin injection is ve in *in vitro* experiments.

n the publications referred to above the ct of heparin had been examined by rminations of the opacity in serum by ns of a colorimeter. Swank, Franklin & stel (1950) and Swank (1951) have rmined the number of chylomicrons, and rved a fall in this after injection of rin, indicative of its antilipemic effect. wn (1950) observed the same and also nd that protamine sulphate can counter- nce this effect of heparin. These studies were made in dogs; in the present investigations we have studied the effect of heparin and protamine sulphate on chylomicrons in man.

## TECHNIQUE

The chylomicrons were counted with the technique described by Frazer & Stewart (1937); for the details of this technique, reference may be made to another paper (Grüner & Hilden, 1953). The tables below give both the total number of chylomicrons, "total", and the number of large, bright chylomicrons, "large".

The fat was administered in the form of 150 ml of a 13 per cent cream. The preparations used were Heparin (Novo) and Protamine Sulphate (Novo).

The experiments were made in subjects who have not presented manifest symptoms of atherosclerosis; "young" means persons under 50 years, "old" stands for persons over 50.

## RESULTS

The effect of heparin on the number of chylomicrons after ingestion of fat in 4 young and 4 old subjects is shown in Table I. The chylomicrons were counted $2^1/_2$ hours after the ingestion of fat; this was followed by an intravenous injection of 150 mg of

Table I

| | | 2½ hours after Ingestion of cream | | Time after heparin injection | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 5 minutes | | 1 hour | | 2.5 hours | |
| | | Total | Large | Total | Large | Total | Large | Total | Large |
| Young | 1. | 44 | 30 | 6 | 5 | 6 | 4 | 1 | 1 |
| | 2. | 87 | 54 | 15 | 5 | 7 | 6 | 4 | 3 |
| | 3. | 94 | 63 | 9 | 7 | 7 | 3 | 7 | 3 |
| | 4. | 78 | 54 | 30 | 13 | 8 | 4 | 7 | 3 |
| Average | | 76 | 50 | 15 | 8 | 7 | 4 | 5 | 3 |
| Old | 1. | 45 | 19 | 10 | 6 | 13 | 4 | 11 | 5 |
| | 2. | 77 | 49 | 20 | 14 | 6 | 2 | 18 | 6 |
| | 3. | 48 | 30 | 7 | 6 | 5 | 4 | 8 | 4 |
| | 4. | 51 | 40 | 8 | 6 | 5 | 2 | 4 | 2 |
| Average | | 55 | 35 | 11 | 8 | 7 | 3 | 10 | 4 |

Effect of 150 mg of heparin in intravenous injection on chylomicronemia 2½ hours after ingestion of cream in 4 young and 4 old subjects.

heparin, and the chylomicrons were then counted 5 minutes, 1 hour and 2½ hours after the injection. The average figures found in the 4 young and the 4 old subjects are given in Figures 1 and 2, compared with the spontaneous variations in the number of chylomicrons after ingestion of fat in the two age-groups (Grüner & Hilden, 1953). It appears that heparin reduces the postalimentary chylomicronemia very considerably in both age-groups.

An intravenous injection of 150 mg of heparin was given simultaneously with the administration of fat in 4 young and 4 old subjects. The results are given in Table II which shows that heparin was capable of preventing the postalimentary chylomicronemia in both age-groups, as counting 2½ hours after the ingestion of fat showed no rise in the number.

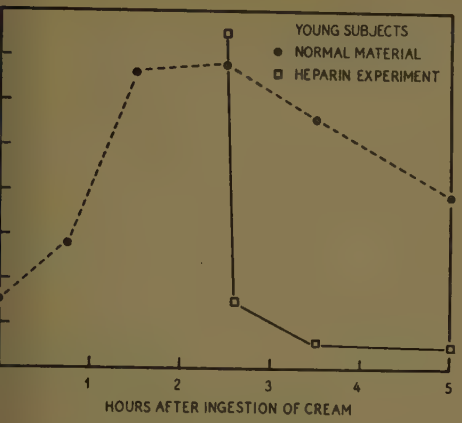
As no difference between young and old was observed in the experiments mentioned above, no such distinction has been made in the following experiments.

Table II

| | | Fasting subject | 2½ hours after ingestion of crea |
|---|---|---|---|
| | | Total | Total |
| Young | 1. | 26 | 15 |
| | 2. | 9 | 2 |
| | 3. | 11 | 7 |
| | 4. | 10 | 8 |
| Old | 1. | 8 | 8 |
| | 2. | 8 | 4 |
| | 3. | 12 | 7 |
| | 4. | 10 | 4 |

Effect of 150 mg of heparin in intravenous in jection simultaneously with the ingestion of crea in 4 young and 4 old subjects.

The doses of heparin mentioned abo were large; we have used a small hepar dose, 20 mg in intravenous injection, in subjects, and Table III shows that this dos about one-eighth of the first, is just as effe tive. Therefore we realized that we wou




. The effect of intravenous injection of hepa-
n the total number of chylomicrons 2½ hours
ingestion of 19.5 g fat in four young subjects.

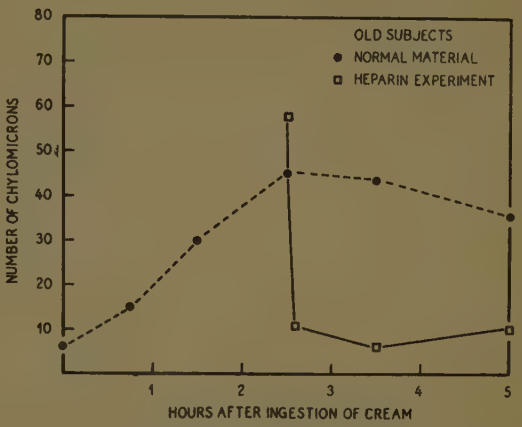
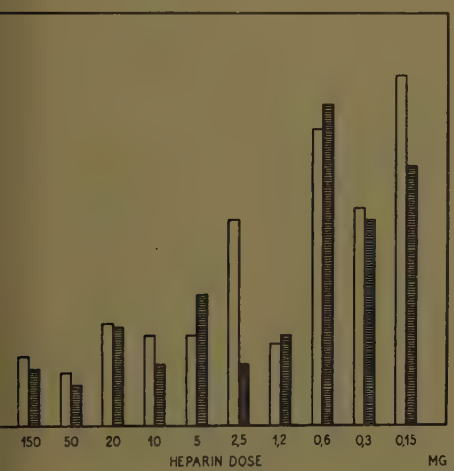



Fig. 2. The effect of intravenous injection of hepa-
rin on the total number of chylomicrons 2½ hours
after ingestion of 19.5 g fat in four old subjects.




. The effect of varying amounts of heparin
ıe chylomicrons 2½ hours after ingestion of
The columns show the per cent left five minu-
fter the injection of heparin. ☐ total. ▤ large.

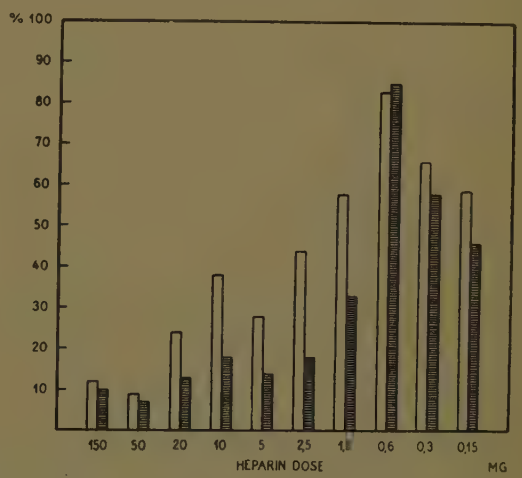



Fig. 4. The effect of varying amounts of heparin
on the chylomicrons 2½ hours after ingestion of
fat. The columns show the per cent left 30 minutes
after the injection of heparin. ☐ total. ▤ large.

to use much smaller doses in the ex-
ation of the relation between the heparin
and the antilipemic effect. Figures 3
4 show the effect of decreasing doses
parin; two experiments were made with
each dose, and the columns in the figures represent the average of the two experiments. It appears that even doses of 1.2 mg exert a distinct effect 5 minutes after the injection. With doses under 1 mg the effect

Table III

| | 2½ hours after ingestion of cream | | Time after heparin injection | | | |
|---|---|---|---|---|---|---|
| | | | 5 minutes | | 1 hour | |
| | Total | Large | Total | Large | Total | Large |
| 20 mg of heparin 1. | 67 | 36 | 20 | 7 | 13 | 6 |
| 2. | 65 | 38 | 14 | 8 | 17 | 4 |
| 3. | 47 | 37 | 12 | 7 | 11 | 7 |
| 4. | 61 | 42 | 14 | 4 | 9 | 3 |
| Average | 60 | 38 | 15 | 7 | 13 | 5 |
| 150 mg of heparin averages from Table I. | 66 | 43 | 13 | 8 | 7 | 4 |

Effect of 20 mg of heparin in intravenous injection on chylomicronemia 2½ hours after ingestion of cream in 4 subjects.

Table IV

| | Fasting subject | | 2½ hours after ingestion of cream | |
|---|---|---|---|---|
| | Total | Large | Total | Large |
| 150 mg of heparin, averages from Table II | 12 | — | 7 | — |
| 50 mg of heparin 1. | 19 | 10 | 41 | 24 |
| 2. | 10 | 5 | 14 | 5 |
| 10 mg of heparin 1. | 5 | 2 | 90 | 59 |
| 2. | 7 | 4 | 78 | 54 |

Effect of heparin in varying doses injected simultaneously with the ingestion of cream.

is doubtful. Half an hour after the injection the effect of the small doses has apparently been reduced to some degree; it seems as if the effect on the large chylomicrons is most persistent. In estimating the values half an hour after the injection it should be borne in mind that a certain spontaneous fall in the number of chylomicrons may have occurred.

Furthermore we examined the effect of different doses of heparin given simultaneously with the ingestion of cream (Tab IV). It appears that — as shown in Table — 150 mg of heparin can completely preve the postalimentary chylomicronemia, 50 m can reduce it considerably, whereas a do of 10 mg is apparently without any effe The effect of the smaller doses of hepar thus subsides, or has ceased completely, the 2½-hour counts.

Heparin was given in intramuscular jection in a smaller experimental ser

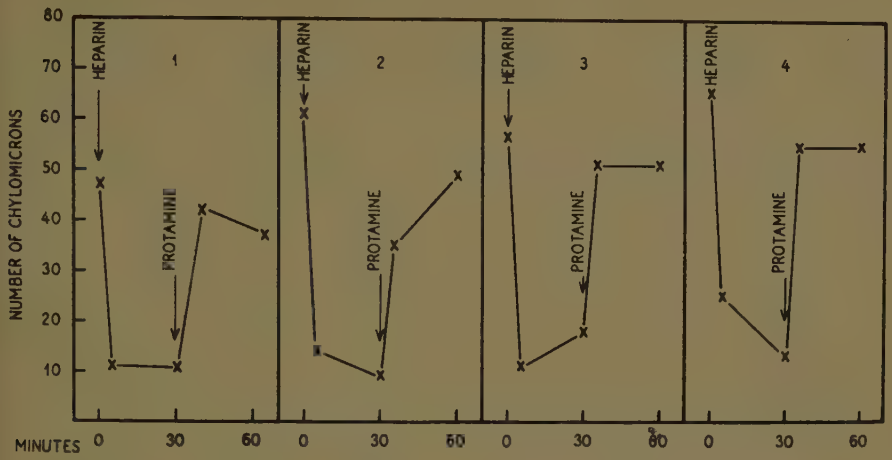



Fig. 5. The effect of intravenous injection of protamine sulphate on heparin depressed chylomicronemia.

Table V

| | | 2½ hours after ingestion of cream | | | |
|---|---|---|---|---|---|
| | | With heparin | | Without heparin | |
| | | Total | Large | Total | Large |
| Young | 1. | 9 | 4 | 94 | 63 |
| | 2. | 42 | 21 | 78 | 54 |
| Old | 1. | 25 | 13 | 48 | 30 |
| | 2. | 31 | 15 | 51 | 40 |
| Young | 1. | 16 | 6 | 64 | 28 |
| | 2. | 84 | 62 | 70 | 39 |
| Old | 1. | 30 | 20 | 25 | 11 |
| | 2. | 50 | 32 | 27 | 14 |

fect of heparin in intramuscular injection 1 after ingestion of cream on the 2½ hour value ylomicrons.

ble V). The intramuscular injection was n 1 hour after and the chylomicrons e counted as usual 2½ hours after the stion of fat. The table also gives the es for the same persons 2½ hours after ngestion of cream when no heparin was n. It appears that doses of 50 mg produce a certain moderate effect, whereas those of 20 mg are ineffective.

It has been reported by Litwins, Vorzimer, Sussman, Applezweig & Etess (1951) that heparin can be resorbed perlingually; this could not be confirmed by Refn & Raaschou (1952) and McDewitt, Huebner & Wright (1952). As the antichylomicronemic effect is apparently a very sensitive test for the presence of heparin in the blood, we have examined whether it would be possible in this manner to demonstrate a resorption of heparin from the mouth. 100 mg of heparin was placed under the tongue in 4 young subjects simultaneously with the ingestion of cream; the 2½-hour values were between 70 and 99 for "total" and between 28 and 91 for "large" chylomicrons; these figures are so high that it can be concluded that the dose of heparin has not exerted any effect.

The effect of protamine sulphate will be mentioned in the following. An intravenous injection of 20 mg of heparin was given in 4 subjects 2½ hours after the ingestion of

Table VI

| | 2½ hours after ingestion of cream | | After heparin injection | | | | After protamine injection | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 5 minutes | | 30 minutes | | 5 minutes | | 30 minutes | |
| | Total | Large | Total | Large | Total | Large | Total | Large | Total | Large |
| 1. | 47 | 37 | 12 | 7 | 11 | 7 | 42 | 31 | 37 | 29 |
| 2. | 61 | 42 | 14 | 4 | 9 | 3 | 35 | 22 | 49 | 34 |
| 3. | 57 | 40 | 11 | 2 | 18 | 7 | 52 | 32 | 52 | 31 |
| 4. | 68 | 48 | 25 | 3 | 13 | 1 | 55 | 35 | 55 | 40 |

Effect of protamine sulphate in intravenous injection on heparin-depressed chylomicronemia afte ingestion of cream.

Table VII

| | 2½ hours after ingestion of cream and heparin injection | | Time after protamine injection | | | |
|---|---|---|---|---|---|---|
| | | | 5 minutes | | 30 minutes | |
| | Total | Large | Total | Large | Total | Large |
| 1. | 5 | 1 | 17 | 3 | 45 | 28 |
| 2. | 0 | 0 | 21 | 14 | 21 | 10 |
| 3. | 13 | 2 | 39 | 19 | 55 | 33 |
| 4. | 1 | 0 | 22 | 10 | 27 | 9 |

Effect of protamine sulphate in intravenous injection on heparin-depressed chylomicronemia afte ingestion of cream.

cream and, after samples had been taken 5 and 30 minutes after the injection, an intravenous injection of 100 mg of protamine sulphate was given, after which samples were taken again 5 and 30 minutes later. Table VI and Figure 5 show these experiments; it appears that protamine sulphate can obviate the heparin effect almost completely. The reason why the values after protamine sulphate do not rise as far as to the level before the heparin injection is hardly that the dose of protamine sulphate has been too low, as it was large in comparison with that of heparin; it should here be borne in mind that the spontaneous curve of the postalimentary chylomicronemia is declining after 2½ hours.

Table VII shows 4 experiments in which an intravenous injection of 150 mg of h parin was given simultaneously with t ingestion of cream; 2½ hours later, wh the number of chylomicrons is very l owing to the heparin effect, an intraven injection of 100 mg of protamine sulph was given, after which samples were tak 5 and 30 minutes later. It appears that p tamine sulphate produces a marked rise the number of chylomicrons.

Finally, we examined whether protami sulphate is capable of changing the numb of chylomicrons in fasting individuals a after ingestion of cream. The results of 8 periments in which an injection of 100 of protamine sulphate was given to fasti subjects are shown in Table VIII. The fa ing chylomicron values rise after the

Table VIII

| Fasting subject | | Time after protamine injection | | | | 2½ hours after ingestion of cream | | Time after protamine injection | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 5 minutes | | 30 minutes | | | | 5 minutes | | 30 minutes | |
| Total | Large | Total | Large | Total | Large | Total | Large | Total | Large | Total | Large |
| 6 | 2 | 11 | 9 | — | — | 41 | 33 | 50 | 34 | 63 | 31 |
| 7 | 4 | 8 | 3 | — | — | 31 | 21 | 33 | 26 | 41 | 29 |
| 9 | 6 | 13 | 6 | 32 | 17 | 34 | 23 | 32 | 21 | 11 | 3 |
| 7 | 5 | 11 | 4 | 13 | 6 | 45 | 35 | 40 | 27 | 46 | 34 |
| 4 | 2 | 8 | 5 | 9 | 4 | 38 | 30 | 43 | 28 | 57 | 43 |
| 4 | 2 | 12 | 7 | 10 | 3 | 53 | 38 | 51 | 36 | 54 | 43 |
| 11 | 3 | 8 | 2 | 2 | 1 | 61 | 46 | 82 | 66 | 91 | 78 |
| 4 | 2 | 13 | 6 | 6 | 3 | 60 | 45 | 49 | 37 | 48 | 34 |
| ean: 6.5 | 3.3 | 10.5 | 5.3 | (12) | (5.7) | 45 | 34 | 47 | 34 | 51 | 37 |

Effect of protamine sulphate in intravenous injection on the number of chylomicrons in fasting jects and 2½ hours after ingestion of cream.

on of protamine sulphate.[1] Table VIII s the results of experiments in another ıbjects who were given an injection of mg of protamine sulphate 2½ hours r the ingestion of cream; these experi- ts show no definite effect of the in- on of protamine sulphate.

## DISCUSSION

he results found in the present investiga- ; show that heparin exerts a very pro- ıced effect on postalimentary chylo- onemia in man; the number of chylo- ons is generally reduced to from 15 to er cent of that present before the in- on. The effect occurs very soon; in our riments with the heparin effect under ous conditions we chose to take the le 5 minutes after the injection; but ıave observed a distinct effect already nutes after.

Graham, Lyon, Gofman, Yankley, Simonton & White (1951) have ascertained by means of ultracentrifugation that heparin changes the size of the lipoprotein particles of the plasma; the effect occurs by 2 stages: At first the large particles (sf 100 or more) decrease in the course of a few minutes, while the number of the somewhat smaller particles (sf 10—100) increases; the second stage occurs in the course of a few hours, and consists in a gradual conversion of the medium-sized particles to quite small ones (sf 10 or less). The decrease in the number of chylomicrons that can be observed with dark-field illumination represents part of the first stage; chylomicrons constitute only the very largest particles (sf 40,000).

The effect of heparin has been examined in detail by Anfinsen, Boyle & Brown (1952); they found that heparin in conjunction with a substance occurring in certain tissues converts a plasma component into the so-called "clearing-factor"; this, in combination with another plasma component ("co-protein"), will produce the changes of

[1] As the differences between the two mean es are 4.0 for "total" and 2.0 for "large", and e mean errors of these differences are 1.33 and this shows a significance of 98 per cent for " and 88 per cent for "large".

the lipoprotein particles observed. In incubation experiments they moreover found that the tissue-factor is present especially in the heart and the lungs.

In our investigations of the necessary dose of heparin we found that even 1 mg in intravenous injection exerts an antichylomicronemic effect, which is just as pronounced as that of the large doses. To explain this finding, it may be mentioned that Anfinsen *et al.* (1952) consider that heparin combines electively with the above-mentioned "clearing-factor".

With regard to the manner in which the chylomicrons disappear from the plasma after the injection of heparin, several authors have imagined that the large lipid particles crowd together, which has been observed directly in dark-field illumination; we have often seen this also in our experiments. The clots formed in this manner are said to be of such an order of magnitude (16 $\mu$ or more) that they may choke the capillaries. Swank & Wilmot (1952) found support to this assumption, as they found an acute fall in the total content of fat in the plasma after injection of heparin; this was ascertained by means of quantitative determinations of fat. This, however, was found in only one out of the 5 experiments made by the writers; in 4 experiments it could be shown that the fat content migrated from the fat fraction that could be separated by centrifugation to the lower ("dissolved") fat fraction. The above-mentioned investigations by Graham *et al.* (1951) by means of ultracentrifugation also suggest that the effect of heparin is primarily that the larger lipid particles of the plasma are converted into particles of gradually decreasing size.

No detailed investigations of the effect protamine sulphate on the lipoprotein pa ticles of the plasma are available in the lit rature. Our results show that this substan has a decidedly counteracting effect on h parin in man. We have not examined wheth the quantitative ratio between the effect heparin and protamine sulphate in this co nection is the same as is known from t influence of these substances on bloo clotting; our doses of protamine sulpha were large as compared to the quantities heparin used.

We consider that we have demonstrat that protamine sulphate increases the spo taneous number of chylomicrons in fasti individuals, while we have not observed a effect on the high chylomicron figures 2 hours after ingestion of cream. Gofman co-workers (Graham *et al.*, 1951, Jones *et* 1951) have put forward the proposition th a certain heparin activity is of normal occu rence, and that this possibly contributes the normal removal of the postalimenta occurrence of the medium-sized and larg lipoprotein particles, including the visi chylomicrons. The mentioned rise in number of chylomicrons after protam sulphate injections in fasting individu might suggest a certain heparin- or hepar related activity in these persons which be counteracted by administration of pro mine sulphate. That this activity should crease in postalimentary chylomicronemia more doubtful, since, as already mentio we have not been able to demonstrate rise in the number of chylomicrons a injection of protamine sulphate during postalimentary phase.

## SUMMARY

eparin in intravenous injection can al-
: completely suppress and also prevent
alimentary chylomicronemia in man.
arge and small doses exert the same
ct; even doses of 1 mg of heparin have
ery pronounced effect, though it is of
ter duration.
he antichylomicronemic effect of heparin
ıtramuscular injection is less pronounced and very capricious; sublingually, heparin exerts no effect.

Protamine sulphate in intravenous injection is capable of counteracting the antichylomicronemic effect of heparin in man.

Apparently protamine sulphate increases the spontaneous number of chylomicrons in fasting individuals. But it does not seem to increase the high number of chylomicrons present 2½ hours after the ingestion of fat.

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# IMPROVED INSTRUMENT FOR INTERNAL STANDARD FLAME PHOTOMETRY

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In a previous paper we have described a flame photometer of our own construction very suitable for routine use at a hospital, this photometer was operated on two selenium photocells and on the internal standard principle (Hallman & Leppänen 1949, Leppänen & Forsander 1951). It was a simple apparatus and easily constructed. The thresholds of error in potassium and sodium determinations were approximately ± 2 per cent. The need for determining the alkali metals has recently gained importance in hospitals in consequence of the necessity to study some diseases and therapeutic methods. Manufactured flame photometers did not prove suitable as such, and moreover, they are still very expensive. It is only after several improvements and adjustments that they can be used for studies of blood analyses on small specimens. On the basis of the experience obtained with our previous flame photometer we constructed a new apparatus. It differs in many respects from the former. The dissimilarity between these two apparatuses will be dealt with in this article later. Our new flame photometer was proved to function quickly, accurately and reliably. Moreover, this new apparatus ours consumes much smaller amounts substrate, and therefore alkali metal dete minations can even be performed on cap lary blood. On the basis of positive exp rience with our new apparatus, we fe justified in describing it as a type of fla photometer which is easily adaptable f hospital use.

### *Atomiser and burner*

In several types of flame photometer t atomiser and burner are more or less simil The burner is made entirely of glass but the upper part there is a metal jacket, t upper end of which consists of a fla grating made of special metal. This me jacket is closely fixed to the glass part means of asbestos packing. The capilla tube designed to suck the specimen into t atomiser is made long enough to withdr the specimen readily from the test tube. T use of the test tubes for preserving t samples saves space when hundreds of a lyses are made at the same time. Each a lysis uses up about 3—5 ml of the specim the atomising of which takes about 15— seconds. Ordinary illuminating gas was us as combustion gas, under a pressure of abo 40 mm $H_2O$. The pressure of the gas fr

[1] Has received a grant from "Tampereen Kirjapaino-Osakeyhtiö" for the construction of the flame photometer.

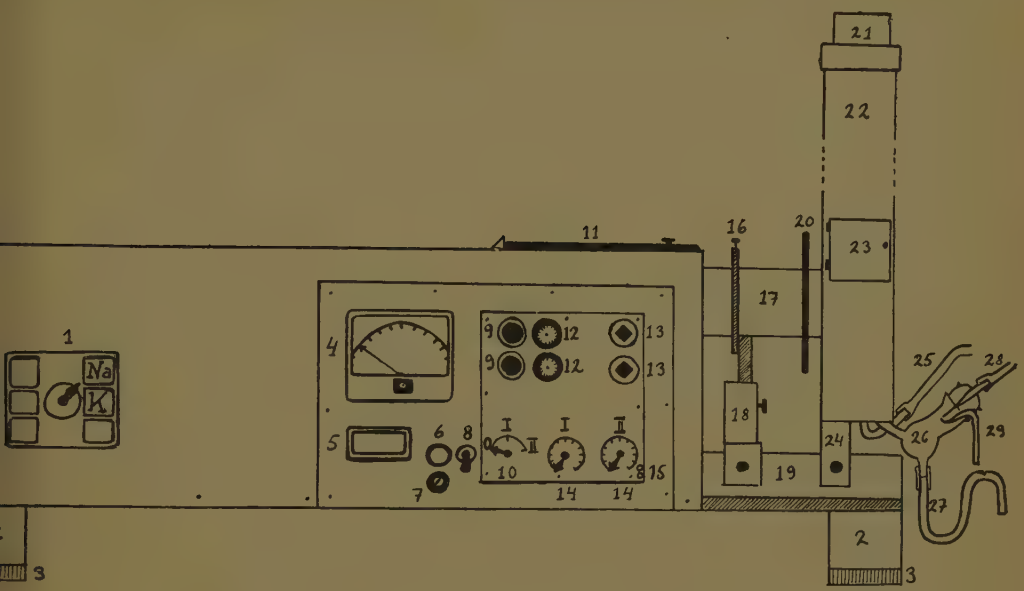


Fig. 1. General survey of the flame photometer.

Wave length (6 different wave lengths).
Foundation of the apparatus.
Porous rubber pieces to prevent vibration.
Galvanometer.
Electric contact (220 V).
Marking lamp.
Fuse.
Switch.
Closer of the photocell aperture.
Breaker for sensitivity areas (I and II).
Lock of the photocell box.
Regulator of the photocell box aperture.
Regulators of the dark current (fine and coarse).
Regulator of sensitivity of the amplifier.
15. Contact for galvanometer to be installed outside.
16. Slit.
17. Connecting tube.
18. Foundation and fastener of the connecting tube.
19. Optic bench.
20. Cooler plate.
21. Cap of burner guard.
22. Burner guard.
23. Shutter for lighting the flame.
24. Foundation and fastener of burner guard.
25. Lighting gas tube.
26. Atomiser.
27. Drain pipe.
28. Air pressure hose.
29. Capillary tube for sucking in the specimen.

ity main remains very constant and the as taken from a compressed air cylinder pressure kept constant by means of lating ventilators. The burner is surded by a metal casing (the "funnel") cooler plates which prevent the heat reaching the electrical part of the apparatus. The flame burns steadily and its size and temperature can be regulated by adjusting the pressure ratio of the air and gas. It is important that the small blue cones of the flame should be regulated quite low on the surface of the grating by means of air pressure.

The box of the atomiser can be opened for cleaning in the place where it is ground joint. The walls of the box are covered with a thin layer of grease, for a better separation of the fluid drops from the wall. In addition, a thin layer of grease on the walls of the glass tubes prevents the formation of undesirable drops in the burner part. About 3 per cent of the specimen to be atomised go into the burner and the remainder escape through the drain pipe. A general survey of the flame photometer is presented in Fig. 1.

*Optical system*

The optical system is illustrated in Fig. 2. Light is taken from the flame (14) on the same side for both photocells. Behind the flame there is a reflex mirror (15) made of stainless steel which reflects and collects the light. The light to be analysed is taken from the flame at its strongest point just upon the surface of the grating for a length of about 3 cm. Thence the light goes through the condenser lens (13), with the source of light in its focus, into the prism (9). In front of the prism there is a narrow slit (12) by means of which the spectrum produced by the prism can be made distinct. At both ends of the prism there are lenses (11 and 4) which serve to regulate the position of the spectrum and its intensity. Before reaching the photocells, the monochromatic light thus obtained is thrown back by means of the mirror (1), in the direction the light has come from, into the photocells (7 and 8). For the lithium light the apparatus is fitted with a special small mirror (2) which is always in the same position directed to the lithium photoelectric cell (7). The larger

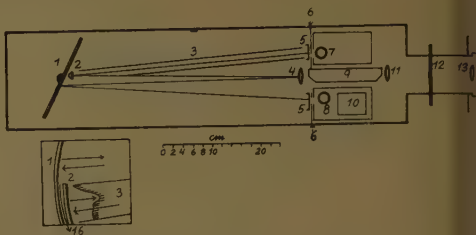

Fig. 2. Optical system.

1. Large reflecting mirror for the light to studied.
2. Small reflecting mirror for the lithium lig
3. Light channel for lithium.
4. Lens.
5. Closer of the photocell aperture.
6. Regulator of the photocell aperture.
7. Lithium photoelectric cell.
8. Photocell of the alkali metals (potassium sodium).
9. Prism.
10. Silica gel box.
11. Lens.
12. Slit.
13. Lens.
14. Burner.
15. Relfex mirror of stainless steel.
16. Black hood for mirror.

mirror (1), by means of which the pot sium and sodium lights are reflected i the measuring photocell (8), can be re lated according to which light is be studied. The regulatory mechanism of mirror has six positions of which, so only two have been in use, one for potassi and one for sodium. By means of the turn regulator of the mirror, the desired spectr can be adjusted on to the photocell. position of each line of the spectrum further defined more exactly by microscr at each point of adjustment. The lower of the large mirror is covered with bl paper (16) up to the upper borderline of lithium mirror, to prevent scattered radia

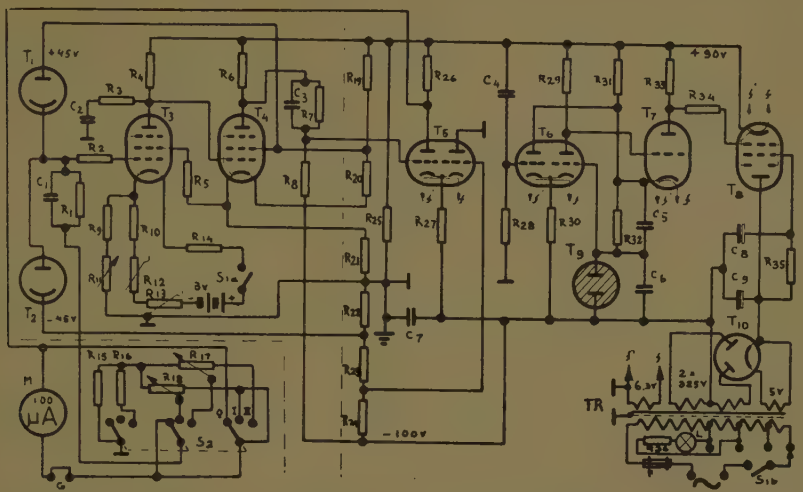

Fig. 3. Specifications of parts in connection with the wiring diagram of the flame photometer.

Resistances:

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 1.0 G ohms | | $R_{10}$ | 200 | K ohms | wire | $R_{19}$ | 5.0 K ohms | 3 W wire |
| 5.1 M » | | $R_{11}$ | 1 | » | » wirepot. | $R_{20}$ | 2.5 » | » » |
| 15.0 K » | | $R_{12}$ | 4×8 | » | » wire | $R_{21}$ | 2.5 » | » » |
| 22.0 M » | | $R_{13}$ | 10×2 | » | » » | $R_{22}$ | 3.0 » | » » |
| 220.0 K » | | $R_{14}$ | 68 | » | » » | $R_{23}$ | 3.0 » | » » |
| 2.2 M » | | $R_{15}$ | 500 | » | » » | $R_{24}$ | 4.0 » | » » |
| 22.0 M » | | $R_{16}$ | 200 | » | » | $R_{25}$ | 7.5 » | » |
| 5.1 M » | | $R_{17}$ | 2 | » | » | $R_{26}$ | 50.0 » | » |
| 3.0 M » | | $R_{18}$ | 10 | » | » | $R_{27}$ | 10.0 » | » |

| | |
|---|---|
| 1.0 M ohms | |
| 0.3 M » | |
| 0.3 M » | |
| 10.0 K » | 6 W wire |
| 10.0 » » | » |
| 0.5 M » | |
| 1.5 K » | |
| 3.3 » » | |
| 200.0 » » | 3 W wire |

Condensers:

| | |
|---|---|
| $C_1$ | 250.0 mm F |
| $C_2$ | 10 000.0 mm F |
| $C_3$ | 10.0 mm F |
| $C_4$ | 0.1 m F |
| $C_5$ | 0.1 m F |
| $C_6$ | 0.1 m F |
| $C_7$ | 0.25 m F |
| $C_8$ | 16×16 m F 450 V E. L. cond. |
| $C_9$ | 16×16 m F 450 V E. L. cond. |

Tubes:

| | |
|---|---|
| 90 CV or 90 AV photocell | $T_6$ 6SC7 |
| 90 CV photocell (Li internal standard) | $T_7$ 6F5 |
| DF 65 D.C. amplifying tube | $T_8$ 6V6GT |
| DL 65 D.C. amplifying tube | $T_9$ 7475 |
| 6SN7 D.C. tube voltmeter | $T_{10}$ 5Y3Gt rectifier tube |

into the lithium channel. The width of the reflected spectrum in the position of the photocell is about 1 cm. In front of the photocells (7 and 8) there are aperture closers (5) for complete closure and movable plates (6) for diminishing the slit. The internal parts of the apparatus are painted in dull black, to prevent any scattered radiation whatever inside the apparatus. The spectrum lines obtained are wide enough but do not interfere with each other. Both the reflecting mirrors are somewhat concave, to prevent too strong a reduction in the intensity of the spectrum light through a long reflexion distance. All the optic parts are firmly fixed to the optical bench, a steel bar triangular in its cross section. For the lithium light the apparatus is fitted inside with a narrow metal channel into which the light can penetrate only by means of the small, *i. e.* the lithium, mirror (2).

### *Electrical system*

The wiring diagram of the electrical system of the flame photometer is presented in Figure 3. The apparatus has two kinds of photocells, Mullard 90 AV and 90 CV, the former having a maximum sensitivity at 4000 Å and the latter at 8000 Å. The relative sensitivy curves of these photocells are illustrated in Figure 4. The amplifying app ratus of the flame photometer is install on the back wall and the galvanometer the front wall. The readings are perform as already mentioned, by the internal st dard method, but an "absolute" method c be used as well, although the possibilities error pertaining to this latter are consid ably greater. The flame photometer has t main sensitivity areas, I and II. Both th areas are divided into 11 sensitivity ar which can be regulated. Figure 5 present typical standard curve on a sensitivity a suitable for the study of blood analyses. T standard curve is linear and the differen between the various dilutions are sufficie pronounced.

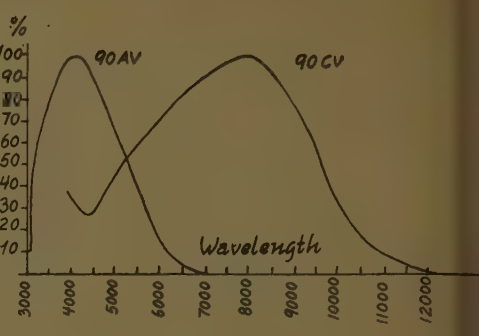


Fig. 4.

### *Stock solutions*

0.2-n KCl and NaCl are used as st solutions from which dilutions suitable use are made. The concentrations of dilutions are expressed in milli-equivale (mEq). The following milli-equival amounts suitable for blood analyses h been used: 0.05, 0.075, 0.10, 0.13, 0 0.20, 0.23, 0.27, 0.30, 0.35 and 0.40. E dilution always has the indentical amo of lithium sulphate, *i. e.* 15 m Mol. P

Fig. 3 (continued).

Miscellaneous:

- $S_1$ 2-pole on-off-switch
- $S_2$ 3×3 pole switch of sensitivity regulator
- M 100 μA moving coil instrument
- G Contact for galvanometer to be installed outside.
- TR Transformer
- L Marking lamp
- F Fuse 1.0 A (5×30 mm)

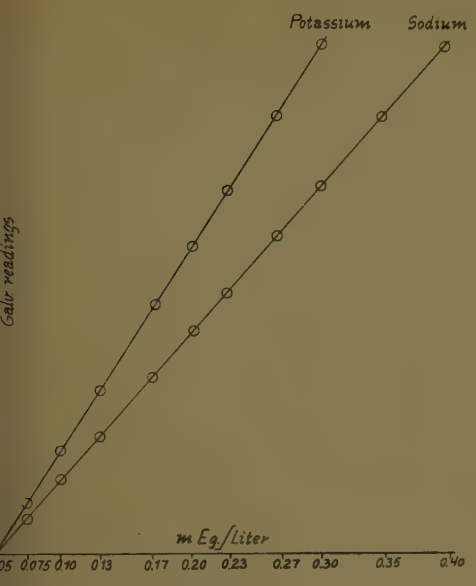


Fig. 5.

each analysis the determination of the ve is performed with al the diluted tions and on the most appropriate sen- vity area. The direction of the curve can accurately determined with several dilu- s. The determination of the curve is formed at least two or three times, until ntical readings are obtained. During the lysis the position of the curve is checked n some of the dilutions already used. instance, every third solution is a test tion.

*Wave lengths*

or potassium 7 669 Å are used, for um 6707 Å and for sodium 5 890/5 896 Å e lengths, respectively. These spectrum s show clearly and do not interfere with n other. Calcium, magnesium and iron do incandesce sufficiently in the flame of ninating gas, therefore they cannot be rmined yet, nor do they cause inter- ference in determinations of other sub- stances. If determinations of calcium, mag- nesium and iron are to be performed, the apparatus would need, by way of spare parts, a different kind of burner and atomiser which would make possible the use of pro- pane and oxygen or acetylene and oxygen for burning.

*Operation and measuring by the internal standard*

Before analysis the amplifier of the flame photometer needs a stabilisation time of 2 to 4 hours. If new batteries and photocells are used, the stabilisation time required can be even longer. There are different photocells for potassium and sodium, and they are exchanged by hand. As a rule, the potassium and sodium determinations are made on different days and as larger series at a time. In the process of the analysis several con- secutive readings can be taken from the same specimen, as one reading takes about 15 seconds and uses up about 3 ml of the spe- cimen. The accuracy is further intensified by the circumstance that the position of the curve in the solution can be rapidly and easily checked after each sample. Potassium solutions and potassium specimens remain unchanged for a longer time than corre- sponding sodium solutions and specimens, since in the course of time alkali can be dissolved into the latter from the glass con- tainers. Glassware of the Pyrex brand is fairly suitable as storage containers for flame photometry. The best glass dishes would of course be those manufactured from insoluble glass. The working rate of our new photometer is about 50 to 60 samples per hour.

Table I

| mEq/l K | Sodium Interference in Potassium Analyses | | | | | |
|---|---|---|---|---|---|---|
| | 0.100 | % | 0.200 | % | 0.300 | % |
| mEq/l Na | | | | | | |
| 0.00 | 0.100 | | 0.200 | | 0.300 | |
| 1.0 | 0.100 | ± 0.0 | 0.194 | − 2.0 | 0.295 | −1.7 |
| 3.0* | 0.103 | + 3.0 | 0.202 | + 1.0 | 0.301 | +0.3 |
| 6.0 | 0.104 | + 4.0 | 0.205 | + 2.5 | 0.307 | −2.3 |
| 10.0 | 0.105 | + 5.0 | 0.207 | + 3.5 | 0.310 | +3.3 |
| 25.0 | 0.109 | + 9.0 | 0.212 | + 6.0 | 0.313 | +4.3 |
| 50.0 | 0.112 | +12.0 | 0.220 | +10.0 | 0.322 | +7.3 |

Table II

| mEq/l Na | Potassium Interference in Sodium Analyses | | | | | |
|---|---|---|---|---|---|---|
| | 0.100 | % | 0.200 | % | 0.300 | % |
| mEq/l K | | | | | | |
| 0.00 | 0.100 | | 0.200 | | 0.300 | |
| 0.01* | 0.100 | ± 0.0 | 0.202 | +1.0 | 0.301 | +0.3 |
| 0.10 | 0.102 | + 2.0 | 0.203 | +1.5 | 0.303 | +1.0 |
| 1.00 | 0.106 | + 6.0 | 0.208 | +4.0 | 0.307 | +2.3 |
| 10.00 | 0.113 | +13.0 | 0.212 | +6.0 | 0.318 | +6.0 |

*General interferences*

Small changes in the combustion gas and air pressure do not cause interferences when measuring by the internal standard method. The lighting gas pressure remains very constant and unchanged. Air pressure can vary between about ± 10 mm Hg, but such a variation is insufficient to disturb the analysis. As already mentioned, the remainder of the metals contained by the blood do not noticeably incandesce in the flame of illuminating gas, therefore their presence produces no interference. The flame photometer was used to examine the effect of potassium and sodium upon each other in those particular concentrations which are resorted to in

Table III

*Effect of interfering substances on 0.20 mEq/l potassium and sodium solutions.*

| Interfering substance | Concentration of interfering substance mMol/l | Concentration obtained Na mEq/l | Concentration obtained K mEq/l | Differen in per ce | |
|---|---|---|---|---|---|
| | | | | Na | K |
| Lactic Acid . . | 0.2 | 0.201 | 0.200 | +0.5 | ± |
| Glycerol . . . . | 1 g/100 ml | 0.198 | 0.201 | −1.0 | + |
| Glucose . . . . . | 1 g/100 ml | 0.200 | 0.200 | ±0.0 | ± |
| Acetic Acid . | 0.2 | 0.204 | 0.203 | +2.0 | + |
| $(NH_4)H_2PO_4$ . | 1.0 | 0.203 | 0.202 | +1.5 | + |
| $CaCl_2$ . . . . . | 0.5 | 0.208 | 0.199 | +4.0 | − |
| $MgSO_4$ . . . . . | 0.2 | 0.200 | 0.200 | ±0.0 | ± |
| $FeCl_3$ . . . . . | 0.5 | 0.202 | 0.202 | +1.0 | + |
| $CuCl_2$ . . . . . | 9.4 | 0.208 | 0.203 | +4.0 | + |
| $MnCl_2$ . . . . . | 0.3 | 0.204 | 0.200 | +2.0 | ± |
| HCl . . . . . | 1.0 | 0.199 | 0.200 | −0.5 | ± |
| $H_2SO_4$ . . . . . | 1.0 | 0.200 | 0.200 | ±0.0 | ± |
| $HNO_3$ . . . . . | 1.0 | 0.202 | 0.200 | +1.0 | ± |

different blood analysis solutions. It w found that the solutions in question do n produce any interference whatever, as th can only appear if the concentration is i creased mainfold. The mutual interferen of potassium and sodium is presented Tables I and II. Each determination w made as five parallel determinations, and t mean value was introduced into the tab The concentrations marked with an asteri can be used in blood analyses. In the sa way a study was made of general int ferences produced by other substanc Table III. Concentrations of 0.200 mEq Na and K were used in the analyses and t amount of interfering components tabulat in the column were added. The analyses we performed as five parallel determinatio and their mean values entered in the tab The alkali metal concentration of each int fering substance was examined and co

Table IV

Precipitation of proteins (potassium)

| precipitated Eq./l potas- n in plasma dilutions | Precipitated mEq./l potassium in plasma dilutions. Precipitation 1 ml conc. $HNO_3$ | Difference in per cent |
|---|---|---|
| 0.106 | 0.104 | — 1.9 |
| 0.102 | 0.100 | — 2.0 |
| 0.110 | 0.110 | ± 0.0 |
| 0.107 | 0.101 | — 5.6 |
| 0.098 | 0.099 | + 1.0 |
| 0.094 | 0.092 | — 2.1 |

Table V

Precipitation of proteins (sodium)

| Unprecipitated mEq./l sodium in cell dilutions | Precipitated mEq./l sodium in cell dilutions. Precipitation 1 ml conc. $HNO_3$ | Difference in per cent |
|---|---|---|
| 0.136 | 0.134 | — 1.4 |
| 0.140 | 0.136 | — 2.8 |
| 0.121 | 0.116 | — 4.1 |
| 0.110 | 0.112 | + 0.9 |
| 0.152 | 0.148 | — 2.6 |
| 0.145 | 0.140 | — 3.4 |

red before beginning the experiment. greatest interference in sodium analyses brought about by calcium and copper, reas in potassium analyses no interfering it is able to affect them to any note- thy degree. The proteins which are ent in the blood do not interfere with determinations. Tests have been made their results are shown in Tables IV V. Concentrated nitric acid was used precipitating proteins. Trichloracetic acid uld not be used in flame photometry, e it greatly interferes with the analysis. protein precipitations were made as five llel determinations and their means have n tabulated.

*paration of the sample. Blood analyses* he blood samples are introduced into the rifuge tube under paraffin. The tube can about 10 ml blood. Heparin, whose al- metal concentration is exceedingly low, sed as anticoagulent. The tubes are al- s centrifuged at the same velocity and the same duration, *i. e.* 3 500 r. p. m. for half an hour. Centrifugation should erformed as soon as posible after with- drawal of the specimen. Plasma is segregated from the red cells and these are centrifuged again at the same velocity, but now for 15 minutes only. Thereafter the plasma which has again become separated is removed from the surface of the cells. The plasma is pipetted in quantities of 1.0 or 2.0 ml into a measuring bottle for determinations of potassium, and for sodium 0.1 to 50 ml. The proteins are not precipitated. The cells are pipetted 1.0 or 2.0 ml into a 50 ml graduate which is filled with water up to the mark and is left until complete hemolysis has occurred. The hemolysed cell solution is pipetted, 5.0 or 3.0 ml for potassium and 20.0 or 10.0 ml for sodium, into a measuring bottle of 50 ml. In each sample to be measured there area lways 15.0 mEq./l lithium sulphate solution. In the handling of cells the proteins are not precipitated either. All solutions to be examined are transparent or only slightly opalescent (potassium solutions of plasma) When studying urine specimens 0.2 to 2.0 ml urine are taken and diluted in a suitable manner to correspond with the concentrations of the dilutions.

When analysing other organic substances

Table VI

0.100 mEq./l NaCl added to plasma sodium dilutions

| mEq./l sodium in plasma dilutions | Present | Found | Difference in per cent |
|---|---|---|---|
| 0.150 | 0.250 | 0.242 | —3.2 |
| 0.142 | 0.242 | 0.244 | +0.8 |
| 0.130 | 0.230 | 0.241 | +4.8 |
| 0.135 | 0.235 | 0.237 | +0.6 |
| 0.120 | 0.220 | 0.225 | +2.3 |
| 0.134 | 0.234 | 0.238 | +1.7 |
| 0.146 | 0.246 | 0.252 | +2.4 |
| 0.129 | 0.229 | 0.220 | —3.9 |
| 0.132 | 0.232 | 0.230 | —0.9 |
| 0.149 | 0.249 | 0.255 | +2.4 |

Table VII

0.100 mEq./l KCl added to plasma potassium dilutions

| mEq./l potassium in plasma dilutions | Present | Found | Difference in per cent |
|---|---|---|---|
| 0.110 | 0.210 | 0.203 | —3.3 |
| 0.102 | 0.202 | 0.198 | —2.0 |
| 0.087 | 0.187 | 0.195 | —4.3 |
| 0.090 | 0.190 | 0.194 | +2.1 |
| 0.080 | 0.180 | 0.181 | ±0.0 |
| 0.105 | 0.205 | 0.200 | —2.4 |
| 0.106 | 0.206 | 0.204 | —0.9 |
| 0.115 | 0.215 | 0.210 | —2.3 |
| 0.118 | 0.218 | 0.214 | —1.8 |
| 0.098 | 0.198 | 0.203 | +2.5 |

the customary combustion or nitric acid treatment is performed according to Lowry & Hastings (1942). Suitable measuring concentrations are arrived at by experimenting. If it is difficult to assess how much alkali metals are contained in the sample to be examined, it is best to make several solutions of one and the same sample, or to perform at the beginning a crude determination with working solutions of high concentration.

Table VIII

Parallel determinations (sodium, plasma)

| Case | Dilution | mEq./l Na in dilution | mEq./l Na in plasma | Deviation | |
|---|---|---|---|---|---|
| | | | | mEq./l | Per ce |
| 1. | 0.1/50 | 0.296 | 148.0 | ± 1.66 | ± 1.1 |
| | | 0.293 | 146.5 | | |
| | | 0.292 | 146.0 | | |
| | | 0.296 | 148.0 | | |
| | | 0.294 | 147.0 | | |
| | | 0.298 | 149.0 | | |
| | | 0.299 | 149.5 | | |
| | | 0.300 | 150.0 | | |
| | | 0.300 | 150.0 | | |
| | | 0.302 | 151.0 | | |

Table IX.

Parallel determinations (potassium, plasma)

| Case | Dilution | mEq./l K in dilution | mEq./l K in plasma | Deviation | |
|---|---|---|---|---|---|
| | | | | mEq./l | Per ce |
| 1. | 0.0/50 | 0.097 | 4.85 | ± 0.085 | ± 1.7 |
| | | 0.096 | 4.80 | | |
| | | 0.099 | 4.95 | | |
| | | 0.096 | 4.80 | | |
| | | 0.096 | 4.80 | | |
| 2. | | 0.102 | 5.10 | ± 0.042 | ± 0.8 |
| | | 0.101 | 5.05 | | |
| | | 0.100 | 5.00 | | |
| | | 0.101 | 5.05 | | |
| | | 0.102 | 5.10 | | |

*Results of the measurements*

The known quantity of 0.100 mEq./l K and NaCl was added to known plasma pot sium and sodium solutions. The results shown in Tables VI and VII. In additi parallel determinations were performed the potassium and sodium quantities. T results will be seen in Tables VIII and I The thresholds of error of the new fla

ometer are about ± 2.0 per cent in ine determinations, but in more accurate rminations the figure of ± 1.0 per cent be achieved.

## DISCUSSION

omparison between the old (Hallman & pänen 1949, Leppänen & Forsander 1) and the new flame photometer. In the ner selenium photocells were used, and electrical impulse produced by them was l directly from the galvanometer without amplifier. However, in the course of e the selenium cells gave rise to numerous urbances, as their efficacy was gradually uced under prolonged use. Moreover, were exceedingly sensitive to humidity temperature. The characteristics of ilar selenium photocells were always htly divergent from each other, and their ability for a flame photometer could only letermined after several had been tested. enium photocells are more apt to retain r efficacy if they can be placed into some ight container, preferably into a small uum. On the other hand, vacuum photo- s are better protected from external in- ences, and for this reason we introduced n into our new flame photometer. thermore, vacuum cells have a more ex- sive and sensitive absorption area, parti- rly if a photocell highly sensitive to two ferent wave lengths is used (4000 and 0 Å). The maximum sensitive area of nium photocells lies between 5000 and 0 Å, and the sensitivity curves show an pt fall at both ends. However, vacuum tocells require an amplifier, as the elec- al impulses produced by them are other- e too weak.

In the old flame photometer glass and fluid filters were used, but their absorption area is fairly extensive, and therefore foreign ions are more apt to cause interference with the measurements. Especially calcium interferes with sodium determinations. In the new apparatus a prism is used, by help of which sharp spectral lines can be obtained. Interference filters can also be used in the photometer, but the potassium light frequently penetrates through these filters causing interference.

In the old flame photometer the atomiser contained compressed air and for the samples two hollow steel needles which were used to atomise the specimen under examination. The fluids were then poured through the funnel from above. In the new flame photometer the atomiser is made entirely of glass, according to Lederle, and the samples are sucked into the atomiser through a capillary tube. The latter method is unquestionably better in flame photometer technique.

In both apparatuses illuminating gas is used for combustion, and it is in fact the best possible gas in determinations of alkali metals.

The curves obtained with the old flame photometer were not linear, which was in particular due to high concentrations of lithium. The curves of the new apparatus are, on the other hand, linear.

Our new flame photometer is of a more complicated structure, but much more accurate and reliable in the performance of analyses.

## SUMMARY

Description is given of a quick, reliable and sensitive flame photometer of the internal standard type.

Its use in the performance of blood analyses is described and the technique of analyses elucidated by means of tables.

A comparison is made between flame photometers functioning on selenium photocells and vacuum photocells.

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# STUDIES IN ARTIFICIAL PERIPHERAL EMBOLISM IN THE RABBIT

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serious risk attending valvulotomy for al stenosis is cerebral embolization. The quencies on record vary from one author nother. Bailey, Olsen, Keown, Nichols amison (1952) observed this complica- in 12 patients out of 235 operated on. er, Brock, Campbell & Wood (1952) d it in 7 of 100 cases, and Harken, Ellis, ter, Farrand & Dickson (1952) reported aths from cerebral embolization in their 30 cases.

ttempts have been made to find means preventing emboli from being carried up the cerebral vesels. Thus Harken *et al.* mmended manual compression of the tid arteries. Bailey *et al.* found such pression inadequate and therefore clam- the innominate artery and ligated the carotid artery for the duration of the acardiac manipulations In none of 80 s in which these vessels had been ped or ligated in this way did cerebral olization occur. Crafoord (1952) used ey's method in 1 case and with success. llen (1952) reported 1 case of death and se of blindness following carotid artery ping in patients with previous cerebral olism.

lready 2 years ago when we started per- ning the operation at Malmö General pital we anticipated this complication and therefore tried to attack the problem experimentally. This work was started and to a great extent finished by the time the papers mentioned were published.

The present paper is concerned with a study of the frequency and distribution of peripheral emboli and an investigation of possible means of minimizing the risk of complicating cerebral embolism in patients submitted to valvulotomy. Four types of experiments on rabbits were performed to study the effect of

a) ligation of the left carotid artery (group II),
b) maintaining the head in a raised position (group III),
c) manual compression of the carotid arteries (group IV),
d) co-effect of maintaining the head raised in association with manual compression of the carotid arteries (group V).

Group I constitutes the control series and was described in a previous publication (Hall, Dencker & Biörck, 1952).

## TECHNIQUE

Three-month old rabbits were used. In these animals the carotid arteries are about 1.5—2 mm in diameter. The animals were anaesthetized with ether or barbituric acid

preparations and mounted on rabbit boards. In those experiments in which embolism was produced experimentally the animals were also heparinized.

"Emboli" with a specific weight of about 1.5 and measuring 0.8×0.8×0.5 mm were prepared from a cellulose sponge with fine pores impregnated with a mixture of lead acetate and glue. The emboli used in group III had a specific weight less than 1.3. The emboli were suspended in heparinized physiologic saline. Some 5—10 ml of the suspension was injected percutaneously in the left ventricle, the number of emboli injected varied from one case to another. In those cases in which the carotid vessels were ligated this was done at the level of the fourth to fifth cervical vertebra. The animals usually survived 1—3 minutes. The animals that survived longer were killed by an injection of air into one of the ear veins.

The distribution of the emboli was afterwards determined radiographically. Radiographs were always taken at two different angles and usually of the entire animal. Only radiographically demonstrable emboli were considered. It was not always possible to trace all of the emboli injected. This may have been due to some of the emboli not being radio-opaque. In some cases two or more emboli may have clumped and thereby appeared in the radiograph as a single one. Emboli located in the heart or lungs were, of course, not considered either, although an occasional embolus in the upper part of the aorta may have been included. As a rule the emboli were easily recognized in the radiograph.

Most of the emboli found in the trunk were localised to the lower section of the aorta and to the femoral arteries. They we more seldom found in other abdomin vessels.

As to emboli in the vertebral artery, only were noted with certainty, one in group and one in group V. In both of these grou the carotid artery had been ligated or con pressed. For technical reasons no attemp were made to distinguish carotid embo from vertebral emboli.

## EXPERIMENTAL

### I. *Distribution of emboli in controls*

Suffice it here to recapitulate an earli report of an investigation of the distribu tion of emboli in rabbits (Hall, Dencker Biörck, 1952). The experimental series co sisted of 14 animals. The number of embo found in a single rabbit ranged from 8 to with a total of 348. The distribution of t emboli is apparent from Fig. 1: 27 per ce were found in the cerebral vessels, 15 p cent on the right side and 12 per cent on t left, 3 per cent in the innominate arter 4 per cent in the right subclavian artery a 3 per cent in the left subclavian artery. T remaining 63 per cent were found in t arterial system below the level of the hea

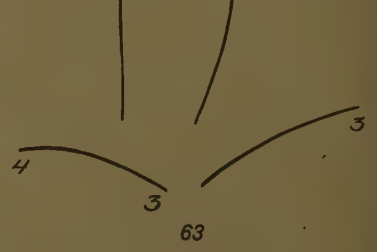


Fig. 1.

*Effect of ligation of the left carotid artery*

his group consisted of 12 rabbits. Al-her 287 emboli were traced, the number single animal ranging from 8 to 44. n the other series, it was not always ble to trace all of the emboli injected. discrepancy was discussed in detail in earlier paper. There is, however, no sible reason to suppose that the distri-n of the missing emboli was not the as that of those traced. In 7 cases e anything up to 11 per cent of the li were not traced, the distribution was tly the same as for the group as a whole per cent of the emboli were found in rain in both series). The distribution of mboli is illustrated in Fig. 2. The right of the brain was found to contain 15 cent, *i. e.* the same as in the controls. innominate artery contained 2 per cent, these were, of course, potential cerebral li. The right subclavian artery con-d 2 per cent, and the left 4 per cent. arterial system below the level of the contained 77 per cent. No emboli were d in the left carotid artery or its ches.

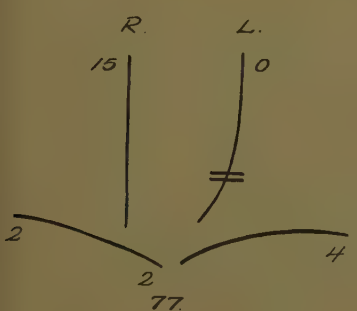


Fig. 2.

III. *Effect of maintaining the head in a raised position*

In these experiments the head of the animal was supported at an angle of about 60° to the horizontal plane, care being taken not to subject the vessels of the neck to any compression or pull. In 2 cases radiographs were made of the skull and the lungs only. Of 19 emboli injected into one of the animals, 3 were found in the cerebral vessels, the corresponding figures for the other animal being 20 and 2. In 2 other cases radiographs were made of the entire body. In these 2 animals altogether 7 emboli were traced in the brain and 40 in vessels elsewhere. Despite the smallness of the groups and the incomplete radiographic investigation, the figures do suggest that cerebral emboli were only half as common in this series as in controls.

IV. *Effect of manual compression of the carotid arteries*

The reliability of manual percutaneous compression was tested in 2 cases. In both about 2 ml mercury was injected into the heart by means of a fine cannula. In controls (3 cases) a striking filling of the cerebral vessels was obtained (Fig. 3). Manual compression of the carotid arteries produced a remarkable reduction in the contrast filling of the cerebral vessels (Fig. 4).

V. *Co-effect of maintaining the head raised in association with compression of the carotid arteries*

In one series a study was made of the combined effect of the last two methods. This group consisted of 7 animals. In 1 case in which 20 emboli were injected, radio-

Fig. 3.

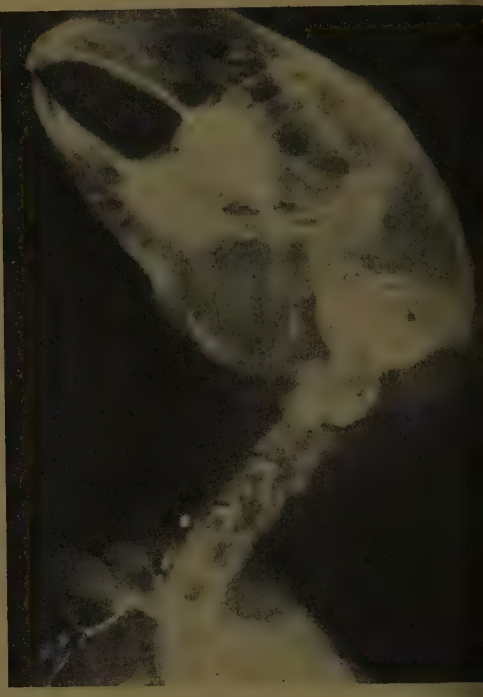

Fig. 4.

graphs were made of the skull and the lungs only. No cerebral emboli were seen. In the other 6 animals altogether 190 emboli were detected in the radiographs, the number in the single animal varying from 5 to 65. In 3 of these 6 animals no cerebral emboli were discernible. In 2 animals 1 embolus was found in the carotid artery at a level below that of the site of the compression. In the seventh case 2 emboli were found in the right carotid artery below the level of the compression and 1 embolus in the left vertebral artery. Thus, of the roughly 200 emboli injected, only 4 were found in the carotid vessels and 1 in the vertebral artery, *i. e.* cerebral embolization in about 2 per cent as against 27 per cent in the controls. In those cases where cerebral embolization w recorded, the lowest total number of em observed in a single animal was 27.

## DISCUSSION

It is difficult to find animals in which vascular pattern in the region of the ao arch resembles that seen in man. There the investigation was carried out on ani in which both the right and the left ca artery branch from the innominate a (Fig. 3). This difference in vascular pa probably had no influence on the res In addition it should be observed that specific weight of the experimental e was probably higher than that of na emboli, although by not more than 0.5

e brain can tolerate fairly prolonged
emia. Bailey *et al.* clamped the innomi-
artery for periods up to 34 minutes
g which they also ligated the left caro-
rtery for 30 to 60 seconds, in some
up to 2 minutes without any persistent
of cerebral anoxia. Weinberger, Gib-
& Gibbon (1940) had shown that dogs
minutes, and Kabat, Dennis & Baker
1) that cats for 6 minutes can tole-
total cerebral anoxaemia without de-
trable alterations in behavior or psychic
ion.

to the present experiments, it was
esting to note that cerebral embolization
red in all of the animals in the control
p and that the number of emboli traced
e cerebral vessels was a high as 27 per
of the total afterwards traced. The per-
ge of emboli found on the left side of
rain did not differ from that found on
ight: this accorded with clinical obser-
ns Hall, Dencker & Biörck, 1952).
me surgeons have contented themselves
ligation of only one of the carotid ar-
s. The efficacy of this procedure was
ed experimentally on the animals in
p II. No increase was observed in the
ntage of emboli in the other carotid
y, in the innominate artery or in the
avian arteries. It was interesting to note
not a single embolus was traced in the
arotid from its origin to the ligation at
evel of the fourth to fifth cervical
bra.

iley *et al.* tried percutaneous manual
ression of both the carotid arteries in
ients. In 2 of them, however, cerebral
lization occurred. Observations made in the present study also show the limited value of this procedure. Judging by the literature, no attempts have been made to decrease the risk of cerebral embolization by supporting the head in a raised position. This was tried, and although the series was too small to permit any valid conclusions, the results suggest that this measure reduces the number of cerebral emboli by about half.

The most interesting observations were made in the group in which manual compression of the carotid arteries was performed in animals with the head raised. In 4 of the 7 animals no cerebral embolus could afterwards be traced. This is all the more striking, because in the other experiments at least some emboli were always found in the cerebral vessels. In the other 3 animals in this group where cerebral emboli were seen, the total number of emboli found in the individual cases was much higher. In this subgroup the number ranged from 27 to 66, in the former from 5 to 30. The percentage of cerebral emboli in the entire group was 2, as against 27 in the controls. These striking results suggest that the method might be of clinical value, especially as a precautionary measure in those cases in which there is no reason to suspect intracardiac thrombosis. The risk of cerebral injury from anoxaemia is also much less than that attending carotid artery clamping. When intracardiac thrombosis is suspected, preference should, of course, be given to Bailey's mehod. The presence of such thrombosis can sometimes be detected by preoperative angiocardiography (Biörck, Axén, Krook, Andrén & Wulff, 1952).

SUMMARY

In a study for possible means of diminishing the risk of cerebral embolization following mitral valvulotomy, radio-opaque emboli were injected into the left ventricle of the rabbit's heart and the subsequent distribution of the emboli assessed roentgenographically.

The observations may be summarized as follows:

1) ligation of the left carotid artery does not increase the risk of cerebral embolization on the other side;

2) maintenance of the head in a raised position or manual compression of the carotid arteries diminishes the risk of cereb embolization;

3) combination of the last 2 metho diminishes the risk of cerebral embolizati to 2 per cent in comparison with 27 per ce in the control group. No cerebral embol was found in 4 of 7 animals in this grou

ACKNOWLEDGEMENT

I am greatly indebted to Dr. G. Biörck, for in ating this work and for criticism of the manuscri I also wish to thank Dr. O. Axén for his help w the radiological part.

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# ELECTROMETRIC METHOD FOR THE DETERMINATION OF CHOLINESTERASE ACTIVITY

## I. APPARATUS AND CHOLINESTERASE IN HUMAN BLOOD

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oxications by insecticides or war gases e organo-phosphorus type are accom- d by an inhibition of the cholinesterase y in plasma and erythrocytes. Thus *itro* determinations of cholinesterase ty in blood have found use in in- ial hygiene, occupational medicine and nedicine. It is, *e.g.*, possible to establish cipient intoxication before any clinically vable symptoms have appeared, or to v the recovery from an intoxication.

veral methods are available for cholin- ase determinations (Delaunois & Casier, Hestrin, 1949, Michel 1949, Strehler eyer, 1952). The Warburg manometric ique is one of the most accurate but res specially trained personel. The pur- of this paper is to give a detailed de- ion of an electrometric technique that its the determination of the cholin- ase activity in six solutions during one This method makes use of the fact acid formation due to the hydrolysis of linester leads to a measurable change H in a buffered solution. To avoid s owing to the variation of enzym ty with pH, the apparatus is con- ed to enable measurements of $\Delta$ pH s of about 0.1 pH unit. A calibration express $\Delta$ pH values as micromoles of acetic acid per ml per minute was published by Tammelin & Löw (1951), and a comparative study of the manometric technique and the present method was made by Tammelin & Strindberg (1952) using plasma and erythrocytes from human blood. In these papers an apparatus with one pair of electrodes was described. A brief description of the present apparatus was given by Tammelin (1953) where the degradation of succinylcholine (Celocurin) was studied.

### APPARATUS

The equipment for the determination of pH consists of the following principal parts:

Six saturated calomel-glass electrode cells which are thermostated in a glycerol bath, an electrically driven cam shaft which connects each of the six pairs of electrodes to a pH meter successively, a pH meter and a recorder (see Fig. 1). The saturated calomel cell is connected to the test solution by six plastic tubes filled with saturated potassium chloride and stoppered by glass needles filled with an agar-agar solution of the buffer used in the determinations of cholinesterase activity (3 ml of Michel's buffer + 3.6 ml of water). The glass electrodes are from Radiometer, Copenhagen, type 202 A. It is necessary to shield the switch between the glass electrodes and the pH meter. The whole switch mechanism is housed, therefore, in a grounded copper chassis. Because of the high internal resistance between glass and calomel electrodes the insulation between the incoming and outgoing leads, on the one hand, and the ground on the other, has

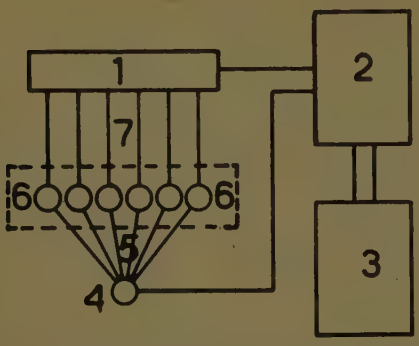



Fig. 1. Block diagram of the electrometric apparatus. 1. Cam shaft. 2. pH meter. 3. Recorder. 4. Calomel cell. 5. KCl bridges. 6. Thermostat. 7. Glass electrodes.

to be particularly effective. The desired result is achieved by ensuring that all leads entering the copper plates lie at a distance of 2 cm from the nearest metal by being sheathed in Plexiglass. The cam shaft is made of glass and the cam wheels are of Plexiglass and have a radius of 2 cm. A model 22 d Radiometer is used as valve potentiometer and the recorder is a shunted Micromax recorder.

To facilitate the exchange of test solutions, the electrodes are mounted in vertically movable electrode holders which rest on the rim of the thermostat vessel.

Fig. 2 shows the apparatus in use. When not in use the glass needles with agar-agar at the KCl bridges are stored in a buffer solution corresponding to their composition and the glass electrodes are kept in distilled water. The measurements are performed in small cylindrical glass vessels with a diameter of 2.7 cm.

The most sensitive parts of the apparatus are the electrodes where irregularities may cause erroneous results. Fluids containing lipids and proteins easily contaminate the electrodes and it is therefore necessary to clean them after each measurement. The glass electrodes were washed with water and careful polished with filter paper. After contamination with lipids they are cleaned with ether. Over the week-end the glass electrodes are kept in 0.1-N hydrochloric acid. In some cases they have been treated with pepsin and hydrochloric acid. Glass electrodes treated as mentioned may kept in use for several months. High concentrati of inhibitors may cause contamination of the ag agar in the glass needles and cause low activit in subsequent experiments. Thus it has been fou convenient to store some agar-agar filled need in buffer solution (3 ml of Michel's buffer+3.6 of water) and to change the needles when a con mination is suspected.

## CONTROL OF THE APPARATUS

A linear relationship between acetic a concentration in the test solution and t registered value on the recorder paper h to be established before use of the apparat This was done by introducing 0.151-N ace acid into a mixture of 3 ml Michel's buff 0.66 ml enzyme solution and 2.90 ml *aq. de* The acid has been introduced in portio

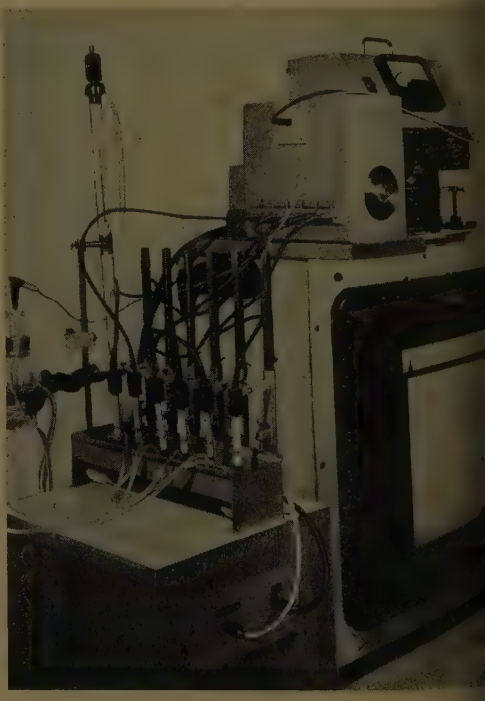
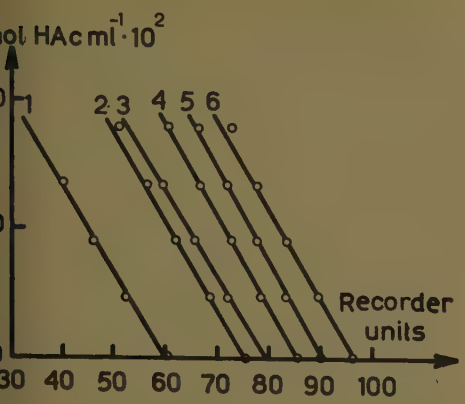


Fig. 2. Electrometric apparatus.




. 3. Calibration diagram for conversion of recorder units to µmol HAc/ml.

an Agla micrometer syringe and after portion the value was registered on the rder paper. The result of a calibration his type of all electrodes is shown in 3.

simpler test for the accuracy of the rodes is to use two buffer solutions with all difference in pH (*e. g.* 7.9 and 7.5) hich case the six pairs of electrodes ld give the same difference on the re- er paper.

## SOLUTIONS

chel's buffer has the following composition: M of sodium barbital (4 1236 g) 0.004 $H_2PO_4$ (0.5446 g); 0.60 M KCl (44.730 g); 0.1 N HCl solution and water to I 1. The choline (ACh) iodide solution is made from g in 5.00 ml of redist. water.

e enzyme solutions are prepared from hepa- d centrifuged human blood. The supernatant a is used directly for the investigations. The ells are washed twice with saline and after- hemolyzed by addition of redistilled water ood volume. These stock solutions generally to be diluted before use. With normal human the plasma is usually diluted 1:1 with re-

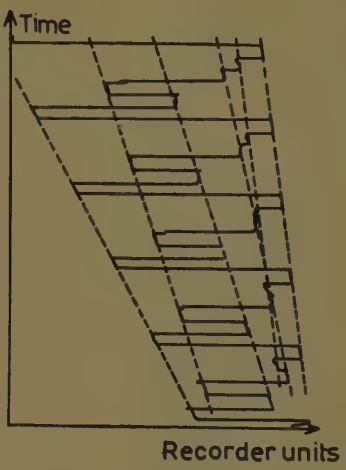



Fig. 4. Diagram obtained as primary result by measurements according to the electrometric method. The continuous line is drawn by the apparatus and dotted lines show activities for the individual solutions.

distilled water and the hemolysate 1:2. The reaction mixture in which the pH determinations are made, has the following composition:

3,00 Michel's buffer solution,
2.34 ml redistilled water,
0.66 ml enzyme solution,
0.60 ml ACh.-iodide solution.

## METHOD OF OPERATION

The buffer, water and enzyme solutions are introduced into the reaction vessels. The electric equipment is connected and the recorded value for each vessel becomes steady in less than thirty minutes. The ACh solution is then added and the enzyme activities in each of the six solutions are recorded as shown in Fig. 4. The activities are obtained as inclinations, expressed as recorder unit per time unit, of the six lines. If absolute values are desired the apparatus has to be calibrated as mentioned above. The diagram

thus obtained (see Fig. 3) and a determination of the velocity of the recorder paper make it possible to express the activity in $\mu$ mol HAc/ml min.

## DISCUSSION

The electrometric method carried out as described has been shown to give the same accuracy as the Warburg manometric technique (Tammelin & Strindberg, 1952) which means that the relative error as a rule does not exceed 5 per cent. This accuracy and t relative convenience of the electrometr equipment recommend the use of this app ratus for routine determinations of choli esterase activities.

## ACKNOWLEDGEMENT

My sincere thanks are due to the Director of th laboratory Professor G. Ljunggren for the ki interest he has shown in this work.

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# ELECTROMETRIC METHOD FOR THE DETERMINATION OF CHOLINESTERASE ACTIVITY

## II. CHOLINESTERASE IN BRAIN TISSUE

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*(Received for publication March 12, 1953)*

ain tissue is known to be relatively rich holinesterases, mostly belonging to the lled specific type (Mendel & Rudney, , Glick, 1945). The specific cholinester- are considered to play an important role only for the transmission across gang- c synapses and from nerve endings to motor end plates of skeletal muscles but across central synapses. The influence of no-phosphorus compounds of the nerve- type on cholinesterase in brain tissue great interest as the mechanism of their iological action has been considered to pendent on their strong inhibiting effect holinesterase.

he purpose of this study was to deter- the useful concentration range for rmination of cholinesterase in brain ogenates using the electrometric method to study the accuracy of the method in kind of determination. Our results de- strate the usefulness of this method with es other than blood. Such an extension he technique may find application in s where the use of blood is not satis- ory, e. g. postmortems.

### MATERIAL AND METHOD

rain tissue was taken from guinea-pigs. animals were killed by decapitation and nguination and the brains were immediately removed. Both halves of the brains were divided in 4 parts, in the cerebrum, the midbrain, the medulla oblongata and the cerebellum, and the parts were put into weighing bottles. They were weighed, and homogenized in a buffer solution in a high-spead homogenizer (Bueler 7073). The volume of buffer solution was calculated according to the following formula: 6 ml Michel's buffer + (6—0.85 A) ml distilled water, where A equals the weight of the brain part. The test solutions used in the electrometric apparatus were prepared from 6 ml of this solution or dilutions. Acetylcholine iodide was used as substrate and 0.6 ml of a solution containing 0.110 g in 5 ml water was added, this giving a substrate concentration in the final solution of $7.32 \times 10^{-3}$ M.

### RESULTS

It is desirable to get high activities in this kind of measurements. However, disturbances presumably due to the high protein content, in this method as in others, limit the concentration of homogenized tissue. A linear relationship between enzyme concentration and activity has to be established in the concentration range used. For this purpose a series of determinations were performed at different dilutions of the brain

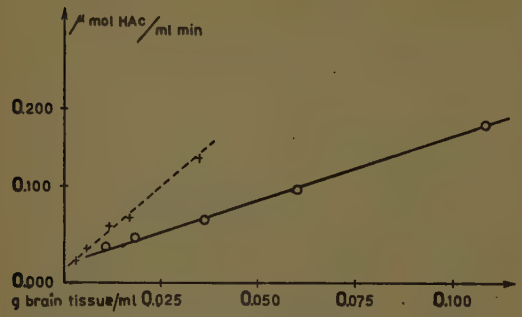


Fig. 1. Cholinesterase activity as a function of brain tissue concentration. Continuous line, cerebrum. Dotted line, medulla oblongata.

homogenisate. The result is summarised in Fig. 1. Thus it has been shown for brain parts between 0.15 and 1.4 g that 3 ml of the basic homogenisate should be diluted with 3 ml of an aqueous solution containing 50 per cent of Michel's buffer.

For the statistical study of the accuracy of the electrometric method, 36 animals were used for 90 determinations during the course of 4 days. Eighteen pairs of duplicate determinations were made on cerebellum. On the other parts of the brain 9 determinations were made on each type. In order to exclude a possible variation among different pairs of electrodes, the different kinds of brain parts were tested with different pairs of electrodes. No difference between the electrodes was found.

The primary values give the following results:

| | $\mu$ mole HAc/ml/min. g. tissue | $\sigma$ |
|---|---|---|
| Cerebrum ..... | 0.494 | 2.4 per cent |
| Midbrain ..... | 1.195 | 2.4 » » |
| Medulla oblongata .... | 1.250 | 4.8 » » |
| Cerebellum .... | 1.855 | 2.4 » » |

The error of the method calculated fr the duplicate determinations on the cerebell parts was found to be 4.7 per cent. The err including biological variation among anim and variations in sectioning techniques w 9.4 per cent.

## DISCUSSION

Most studies of the cholinesterase in bra have been made by means of the Warbu manometric technique. This method has error of about 5 per cent. The present r sults show that the electrometric meth described in part I (Tammelin, 1953), well applicable to determinations of choli esterase in brain tissue. It is relatively co venient and the error of the method do not exceed that of the Warburg techniqu

## ACKNOWLEDGEMENT

Our sincere thanks are due to the Director this Laboratory Professor G. Ljunggren for t kind interest he has shown in this work.

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# ENZYMATIC DETERMINATION OF URIC ACID

## WITH DETAILED DIRECTIONS

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(Received for publication March 14, 1953)

ce Kalckar in 1947 used uricase as a or the spectrophotometric determina- of uric acid, the method has been ed for clinical purposes by one of the rs (Prætorius 1949). Although the dure is very simpel and the specificity, ensitivity and the accuracy are very the method has not been widely used t in the laboratories of some larger als in Scandinavia and a few other . The reason for this is firstly that ecently purified uricase was not gener- vailable, and secondly that the measure- must be made in a spectrophotometer equipment for ultraviolet light.

w highly purified uricase prepared ling to the directions of Holmberg ), somewhat modified by Prætorius ), is on the market,[1] and spectrophoto- in the ultraviolet region is no longer ceptional examination in the clinical tory. A detailed description of the cal problems which, in our experience, s arise to the technician not familiar nalyses of this kind, might be useful. e entering on a description of the d and its modifications for special purposes it was thought advisable to make a survey of the basic principle, the procedure in outline, and the calculation.

### PRINCIPLE

The method is based on the following facts: Uric acid produces a very strong absorption of ultraviolet light. Maximum absorption appears between the wave-lengths of 290 m$\mu$ and 295 m$\mu$. This absorption disappears completely after addition of uricase. The concentration of uric acid in a solution is proportional to the extinction difference before and after the action of uricase. If 1 $\mu$g of uric acid per ml has disappeared, the fall of the extinction at 292.5 m$\mu$ is 0.0745 (light path 10 mm), and accordingly, if the extinction difference observed in the presence of the enzyme is $-\Delta$ E, the concentration of uric acid *in the optic cell* is

$$\frac{-\Delta E}{0.0745}\ \mu g \text{ per ml (formula 1).}$$

#### *Procedure in outline*

In an optic cell, the "measuring cell", a certain volume (m) of the material is diluted by a volume (b) of a buffer at pH 9.3, and the extinction of this dilution at 292.5 m$\mu$ is read against the contents of another cell,

[1] *Iricase-Ido" Ferrosan Export Corp.*, Bleg- ej 72, Copenhagen Ø.

the "reference cell". This reading, the *"before-enzyme reading"*, must remain quite constant for at least 5 minutes and should be fixed between 0.100 and 0.400. (See "Optic cells".)

The "before-enzyme reading" must not be confused with the "zero-time extinction", which is the sum of this reading and the "self extinction" of the uricase added at zero-time.

The zero-time extinction can be obtained in two ways either by extrapolation to zero-time from a series of extinction figures recorded in succession within the first minute or two after the addition of the enzyme to the measuring cell, or by measuring directly the "self-extinction" (see "Self-extinction of the enzyme").

In less than half an hour after the admixture of uricase the extinction has fallen to a final constant level. $-\Delta E$ is the difference between the zero-time extinction and the final extinction.

The concentration of uric acid in the undiluted sample of the material is:

$$\frac{-\Delta E}{0.745} \cdot \frac{m+b}{m} \text{ mg per 100 ml} \quad \text{(formula 2)}$$

## PROCEDURE IN DETAIL

### *Optic cells*

Quarts cells with a light path of 10 mm, and of standard size (10 mm × 10 mm × 50 mm) are preferable for measurements in the ultraviolet region of the spectrum. At 290—295 m$\mu$, however, glass cells can be used without shortcomings, because the absorption at this wave-length is only moderate. At 292.5 m$\mu$ the extinction of a glass cell against a quartz cell will not exceed 0.400. It follows from this that if, in any kind of analysis in quartz cells, the "before-enzyme reading" is between 0.400 and 0.8 this provisional reading can be reduced an optimal figure by replacing the qua reference cell by a glass cell.

### *Slit-width*

With the hydrogen lamp in the prop optimal position, a slit-width of 0.3 mm adequate for analyses of diluted urine, a 0.8 mm slit-width is convenient for analy of diluted serum or plasma from hun blood.

### *Glycine buffer*

The glycine buffer is stored as a str ($^2/_3$ *M*) solution at refrigerator temperatu To avoid contamination by microorganis it is saturated with chloroform, a sm excess of which lies at the bottom of container. This stock solution has to diluted to $^1/_{10}$ before use, and also bef any checking of the pH in a pH-meter.

0.5 litre of the stock solution is m from 25 g crystalline glycine dissolved about 200 ml of $CO_2$-free water; 110 of *N* NaOH are added, and the volume made up to 500 ml with water.

The pH must be exactly adjusted by addition of HCl or NaOH to 9.30[1] should always be measured in the dilu buffer ready for use, ($^1/_{15}$ *N*).

### *Pipettes*

Samples of the material (serum, plas or urine) are most accurately diluted means of constriction pipettes; the Ca

[1] The optimal pH is 9.3—9.4. When the pH be determined with a certainty of ± 0.1, an adj ment to pH 9.3 is preferable. (pH 9.4 + 0.1 co sponds to a much lower enzyme activity pH 9.3—0.1.)

semi-automatic pipettes may be re-
nended for volumes up to 1 ml. Samples
ı ml of serum or stabilized plasma are
ted off to about 1 ml of the undiluted
*M*) buffer in a 10 ml volumetric flask
the volume is made up to 10 ml with
r. In this case the dilution ratio $(\frac{m}{m+b})$
.

quantities of only 0.1 ml of the mate-
re available, the dilution with the buffer
ost easily carried out in the measuring
When this cell contains exactly 3 ml
$_5$ *M* of the buffer 0.100 ml is replaced
.100 ml of the sample. Since $\frac{m+b}{m}$
is case is 30 (see formula 2) the uric
concentration in the undiluted material
mg per 100 ml multiplied by $-\Delta$ E.
simple calculation of the uric acid con-
ation in the material may also be made
ı the dilution is performed in a volu-
ic flask. If 265—270 $\mu$l (exactly 268.5
is diluted to 10 ml, the uric acid con-
ation in the undiluted sample is 50 mg
100 ml multiplied by $-\Delta$ E.[1] Pipettes
volumes of 5—25 $\mu$l are useful for
se suspension. (See "Diluted uricase
..".) For determinations on plasma
les of 20—25 $\mu$l see "Determination on
ll Volumes".

### *Stirring*

the 10 ml volumetric flask neither
ing nor shaking must take place until
lask has been filled up to the 10 ml mark

Carlsberg pipettes of any size are obtainable
Mr. H. Pedersen, Gl. Carlsbergvej 8, Copen-
, Valby.

Fig. 1.

Fig. 2.

with water. After this the stirring cannot be too vigorous.

Stirring in the optic cells should be performed by means of special stirrers of "plastic" or some other material which does not scratch the polished walls. Thin rods with a broad flat end (Fig. 2) of plastic are suitable.

The addition and the stirring of the enzyme in the measuring cell must take place simultaneously, exactly at zero-time, that is, when the second hand of a watch passes zero. This is conveniently done by placing 5—25 $\mu$l of the uricase suspension in a small groove cut for this purpose into the upper side of the flat end of the stirrer.[1] (See "Diluted uricase .....")

[1] Special stirrers for enzymatic spectrophotometry can be obtained from Mr. P. Madsen, Inst. Pharmacology, Juliane Mariesvej 20, Copenhagen, Ø.

*Time recording for extrapolation*

After admixture of the uricase, 3—5 readings should be made as soon as possible, within one or two minutes after zero-time. The time corresponding to a certain extinction figure is, of course, not the time at which the figure is noticed or taken down, but the exact time (seconds) at which the galvanometer needle, which moves from the right to the left, passes the zero position. Therefore, the time must always be recorded without delay, whereas the extinction figure can be noted down at leisure: the galvanometer needle moves, but the extinction scale is at rest!

*Self-extinction of the enzyme. ($+ \Delta E$)*

If the inexperienced technician has not succeeded in obtaining reliable parallel figures for time and extinction to ensure a safe extrapolation of the extinction to zero-time, the zero-time extinction must be calculated as the sum of the "before-enzyme reading" and the "self-extinction" of the employed sample of uricase. This self-extinction is measured in a fresh measuring cell containing the $^1/_{15}$ M glycine buffer, the extinction of which should be read against a reference cell with exactly the same contents. (The volumes of the cell contents should, of course, be identical with that of the diluted sample in the measuring cell for the uric acid analysis).

If the two cells and their contents are identical, the reading of one against the other will give about 0.000. By adding a sample of uricase to the cell in the measuring position the reading will increase. This extinction rise ($+ \Delta E$) is the self-extinction of the used amount of uricase.

Since the optic cells are never *quite* ide tical $+ \Delta E$ should always be calculated the difference between the reading after a before the addition of the enzyme to t buffer. The sum of this self-extinction a the "before-enzyme reading" of the dilut sample of uric acid-containing material the zero-time extinction.

*Final extinction*

If the extinction has been constant for t last one-third of the period since zero-tim further observations are not necessary a the reading may be considered to represe the final extinction. This final reading increased after stirring if the "self-extin tion" of the enzyme has not remained co stant. (For corrections see "Self-extinctio in the following section.)

QUALITIES OF COMMERCIAL URICAS

The following requirements must be f filled by any specimen of uricase to be us for the enzymatic spectrophotometric dete mination of uric acid:

The uricase suspension must have a c tain strength, which must not be essentia reduced by storage at refrigator temperatu The "self-extinction" of an amount requir for an analysis must be moderate compar to the extinction fall which is the measu of uric acid, and the "self-extinction" m be almost a constant figure through an o servation period similar to the reacti period in the analysis.

*Standard activity*

Commercial uricase is as a rule obtain as a rather concentrated suspension, the a tivity of which is adjusted more or l

tly to the so-called "*standard activity*". s means that 2 ml of the commercial ase suspension contains 1000 units of ysis (1 unit = 2 microlitres).

ne *unit* of analysis is the amount of ase which, when added to 3 ml of a fered solution of uric acid, containing $^4/_3$ rogram per ml, produces an extinction ease at 292—293 m$\mu$ of 0.010 in the two minutes, when the analysis is made rding to the directions in this paper. The l extinction will be attained within one from zero-time.

*Diluted uricase suspension for use*

n order to estimate the activity of the mercial enzyme suspesion, the inexperi- d technician should dilute a small portion ml) to 1 : 5 with $^1/_{15}$ *M* glycine buffer. dilute uricase suspension is called the ginner's uricase" (1 unit = 10 $\mu$l). If the mercial uricase has the standard strength, extinction decreases about 0.010 in the two minutes after the addition of 10 $\mu$l ne beginner's uricase to 3 ml of a sample lasma diluted as described above (see ettes").

n cases with a normal plasma uric acid entration, the final value is reached in less than half an hour with one unit. fter a few days' experience the tech- n will want a higher reaction rate. This be obtained by using 20 or 25 $\mu$l of the nner's uricase; if this is not satisfactory r the next step is to use the standard ase diluted 1+1 with the buffer.

the uricase activity is found not to ll the requirements made on a standard uricase, the cause may be that the standard suspension has not been kept at refrigerator temperature or that the buffer used for dilution does not have the right pH (9.3±0.1), or that uricase has been spoiled by contamination with, for instance, oxidizing substances; therefore the pipettes used for withdrawing small samples of the standard uricase for dilution must be thoroughly cleaned. Fuming nitric acid or chromic sulphuric acid must be completely eliminated from the pipettes by thorough rinsing by an stream of water rapidly and repeatedly interrupted by air sucked through the pipette in the direction from the tip towards the constriction (Fig. 1). The water should never be pressed in the opposite direction.

The activity of the standard uricase, cannot be expected always to be the same. If the uricase has been sedimented for weeks or longer periods one will find a lower activity upon resuspension than is observed when the enzyme has been suspended once or more each day for some days. It is, therefore, impossible to give more exact rules for the dilution than those stated above.

*Self-extinction*

The self-extinction of 10 $\mu$l of the "beginner's uricase" must not exceed 0.020 and must not decrease more than 0.003 in half an hour. If a greater fall is found it must be subtracted from $-\Delta E$ (see "Principle" — formula 1).

A second rate specimen can be used, only if this time consuming correction is made according to the above directions (see "Self-extinction of the enzyme").

It is very important to keep in mind that the uricase should always be suspended, preferably half an hour before use, and not resuspended before sampling. Although the fairly large particles have sedimented in half an hour the activity of the uricase suspension is not reduced, — on the contrary. If a technician stirs the suspension immediately before the self-extinction will be higher, and, what is worse, it may not remain constant. Hence, a single shaking of the uricase container $^1/_2$—1 hour before use and no resespension, except when samples are not needed within 30 minutes, musf be considered important precautions, especially when the enzyme has been more or less inactivated by neglect (standing for hours at room temperature or contamination).

## DETAILS OF BLOOD ANALYSIS

The dilution of serum or plasma is described in the section on "Pipettes". The measuring cell ordinarily holds 3 ml and the dilution ratio $\frac{m}{m+b}$ is about 1 : 30 or between 1 : 30 and 1 : 40. If these serum or plasma dilutions are read at 292.5 m$\mu$, the extinction against a reference cell containing water or buffer solution will be 0.700—1.000. When the read extinction, the "provisional reading", is about 0.700 it may be reduced by replacing the quartz reference cell by a glass reference. (See "Procedure in outline".)

For routine analyses it is simpler, however, to make a dilution of plasma or serum in the same manner as when preparing the dilution for the measuring cell. The reference dilution should contain only about 80 per cent of the amount of plasma o serum present in the dilution for the measu ing cell. This means that when the dilutio takes place in the cells, with 100 $\mu$l of plasm in the measuring cell, the amount of plasm for the reference cell is obtained by measu ing off only about four-fifths of the pipet volume. When the dilution is carried out i volumetric flasks, with 268—269 $\mu$l o plasma in the measuring cell, the amount plasma for the reference cell dilution ma be about 0.2 ml.

In serial determinations the technician wi soon realize that different sera and plasma do not produce the same extinction eve though they are read against the same r ference. If the "before readings" are b tween 0.100 and 0.400 no difficulties wi rise. If the "before reading" is higher tha 0.500 the reading accuracy is not very hig and it is advisable either to use a referen containing a little more blood, for instan four-fifths of the same plasma as in t measuring cell *concerned,* or to replace t common quartz reference cell by a glass c with the same contents.

Before choosing a common reference it wise to read the measuring cells (containi different sera) against a water referen Then a reference of the serum with t *lowest* extinction is made by means of fou fifths of the serum in the dilution for an lysis. Otherwise the "before readings" sera with a low absorption may be low than 0.100 or even negative, so that the e zymatically produced extinction decrea cannot be read on the extinction scale, least not with the reference dilution e ployed.

in any case the final reading gives a
ıtive figure, the — $\Delta$ E is, of course, the
of the extrapolation figure and the
ierical value of the final reading. The
lt is not less accurate because the final
re is read in the negative part of the
.

Vhen the final reading is so low that it
eyond the negative end of the scale, the
1E can nevertheless be obtained by using
"auxiliary reference", *i. e.* a random
tion, in a reference cell against which
final extinction can be read within the
ge of exact measuring (between 0.400
minus 0.040).

)ne never fails if the serum concerned is
l as a reference in an amount of four-
hs of that used for the analysis as men-
ed above. The read "auxiliary final ex-
tion" is, of course, too high, when the
rapolation is made against a "darker"
erence. The figure to be subtracted from
auxiliary final extinction is the extinc-
of the original reference as read against
auxiliary reference cell.

TERMINATION ON SMALL VOLUMES

f it is necessary to measure the uric acid
centration in samples of 20—25 mikro-
es, the dilution must take place in the
ısuring cell by adding the available
ount to 1000 microlitres of buffer. It is
uch cases necessary to insert a diaphragm
he spectrophotometer with an opening of
mm, and it must be ensured that all the
t from the opening passes through the
tents of the *measuring* cell, *i. e.* the bot-
of the cell must be raised by means of

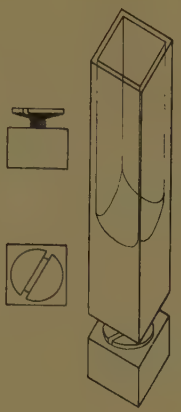

Fig. 3.

an ebonite or wooden block between the bottom cell holder and the bottom of the cell. The position of the cell can best be correctly adjusted by means of a screw with a big flat head. (Fig. 3.)

The calculations must be carried out by means of formula 2.

## DETAILS OF URINE ANALYSIS

It is, as a rule, most expedient to dilute specimens of urine in 10 ml volumetric flasks, as in the case of blood plasma. The volume of urine, however, must be 25 $\mu$l in all cases of measuring on 24-hour urine and also on most samples collected through shorter periods.

If the urine is very rich in uric acid 10—15 $\mu$l of urine per 10 ml will be better, and if the urine is highly diluted, 100 $\mu$l of urine per 10 ml is necessary to obtain a high accuracy.

The reference cell may contain water or buffer.

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# PARTITION CHROMATOGRAPHY OF THE PORPHYRINS

## A NEW APPARATUS

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### INTRODUCTION

Nicholas & Rimington (1949) described n invaluable technique for the qualitative nalysis of the porphyrins by paper partition hromatography. They used a 35 × 6 cm aper dipping into a glass trough containing :4/2:5 lutidine saturated with water. 'aper, solvent and trough were all enclosed n the conventional glass museum jar with iding vaselined lid.

The apparatus to be described is a new ne for carrying out the Nicholas—Riming- n technique, and has some advantages over ie conventional design in that it is simple nd inexpensive; it can be set up in batteries the same cupboard for simultaneous ana- ses of twelve samples under identical onditions; it employs a very long paper and ives very reliable $R_F$ values, and it can be ade entirely in the laboratory.

### METHOD OF CONSTRUCTION

Each unit consits of a tube 35″ long, of on-fluorescent glass, eg: Hysil, 2 1/2″ in ameter, with compressed-cork stoppers at oth ends. The stopper which is to be at e top of the unit is bored right through centrically with a 3/8″ hole, and then h ay through on the same centre (from the narrow end) with a 5/8″ hole. The holes are carefully filed to remove any roughness. (Fig. 2.)

The trough is made by cutting a window 5/16″ wide horizontally across one side of a flat 50-tablet Aspirin bottle, 3/8″ below the shoulders. This is done easily with a dental drill, using a thin carborundum wheel in the handpiece, and keeping it lubricated with warm water.

The trough is carefully screwed into the 5/8″ hole in the stopper (using the thread already on the glass neck), and a short glass tube fitted into the 3/8″ hole so that it projects down into the trough just past the window. The upper end is closed with a cork.

The unit is now complete, and six of these units are supported vertically by large Terry clips in a cupboard 5ft × 2ft × 1 1/2ft (Fig. 1). There is a fan in the bottom of the cupboard for air circulation, and above this, screened from the units by an asbestos baffle, a 16 C. P. carbon-filament lamp for heating, controlled by a Sunvic TS 3 thermostat near the roof, with a relay. There is a thermometer at the side. This cupboard, which is thus an incubator, is closed in front by an inner glass door, and an outer wooden ne. It is therefore possible to view the units at any time with the ultra-violet lamp or

Fig. 1. General view of apparatus.

with the unaided eye, without interfering with the temperature of the cabinet.

The cabinet is screwed to the floor for stability.

## TECHNIQUE

The following description refers particularly to porphyrin work, but can be readily adapted for any other type of paper partition chromatography in one dimension

Before each experiment, the air in each unit is saturated with water and lutidine vapour at the temperature of the cabinet — usually 24° C — by placing a small beaker of water at that temperature, containing also a wide mouthed tube of lutidine, in the bottom of each unit, resting on the stopper. This should be done one hour before the start of each run.

Whatman No. 1 filter paper can be supplied in rolls 1¼" wide. For each unit a length of 30" is cut off, and these lengths are marked 3" from one end with a horizontal line. The porphyrins are spotted across the centre of the line, or if desired two spots may be placed side by side. The porphyrins are usually spotted from lutidine solution. When the spots are dry, the end of the paper near the spots is folded ½" from the end upwards, and then 1" or so (depending on the height of the window from the bottom of the trough) from this fold downwards. The end fold is the

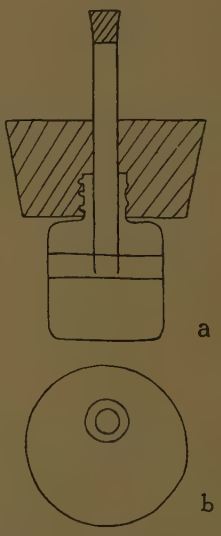


Fig. 2. a. Diagram of head showing method of fixing trough.

b. Diagram of lower end of stopper showing method of boring holes.

ıshed through the window of the trough, ıd down to the bottom, an "anchor" of ıass rod being added to keep it in place. he head, complete with the paper is then ıarefully inserted into the unit-tube, and hen all units are complete, lutidine satuıted with water at the temperature of the ın — 24° C — is added through the small ıbes, so that it fills the trough to a depth f about 1/4" below the window, and the mall corks are replaced. The doors are osed gently, and the apparatus left for 18 24 hours.

## COMMENT

This apparatus was used in the investigaon of the porphyrin of the integument of sterias rubens L. (Kennedy & Vevers 953 a. b.)

The apparatus has now been made entirely f glass by Messrs Quickfit and Quartz, taffordshire, England, and I am considerbly indebted to them for the way in which ıey have carried out the work. The all-glass ead of a unit is shown in Fig. 3.

Nicholas & Rimington (1951) reported ıat they no longer found it necessary to use ı atmosphere of $NH_3$ vapour in their ıromatograms, as they originally described ı 1949. The $R_F$ values are slightly lower ı the absence of $NH_3$.

## SUMMARY

A new apparatus is described for the oneimensional paper partition chromatography f porphyrins using the Nicholas—Rimingon technique.

Fig. 3. Head of all-glass apparatus.

The apparatus is simple and adaptable to other investigations. It embodies advantages over the conventional types of apparatus, foremost among which is the possibility of setting up a large number of samples at once under identical conditions. The apparatus can be made in all-glass form.

## ACKNOWLEDGEMENT

I am grateful to Mr. S. H. Rotherham, of my laboratory, for making the cabinet, to the firm of Quickfit and Quartz for their careful manufacture of the all-glass unit and to Mr. John Gray of the Department of Forensic Medicine for the photographs.

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# ESTIMATION OF PORTAL VENOUS PRESSURE BY OCCLUSIVE HEPATIC VEIN CATHETERIZATION

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Surgical treatment is now widely ad-ocated in the management of hematemesis nd recurrent ascites in cases of portal ypertension. The establishment of a porta-aval shunt for relieving the increased ten-on of the portal vein and its radicals still anks as the standard operation, but pro-ising results seem to have been achieved y the later and simpler procedure of ligat-g the hepatic artery and the splenic artery Rienhoff, 1951). The value of this latter ethod still remains to be definitely settled.

In the establishment of the diagnosis and the assessment of indications for surgical tervention by either method as well as in e evaluation of the endresults of operation, nowledge of the degree of portal hyper-nsion is important. Until quite recently ortal tension could only be measured after parotomy, and even then it was not certain at the pressure recorded at the operating ble was a true measure of that present nder ordinary conditions, as it is not known hether the anesthesia and the opening of e abdominal wall may influence portal nsion, which therefore had to be judged relation to simultaneous measurement of e systemic pressures.

The degree of portal tension has been deduced from the pressure of the abdominal collaterals (Davidson, Gibbons & Faloon, 1950) and from the tension of hemorrhoidal veins or oesophageal varices (Allison 1951, Palmer 1951). The former method, however, can only be used when collateral veins are available for measurement, and the latter method seems to be technically difficult and risky.

In 1951 Myers & Taylor described a new method for measuring portal tension. The method was suggested by the observations made by Hellems, Haynes, Gowdy & Dexter (1948) when measuring the pulmonary capillary venous pressure (PCV). The pressure recorded via a catheter occluding a peripheral branch of the pulmonary artery was found to be almost identical with the pressure in the small veins of the lung. (Hellem, Haynes, Gowdy & Dexter 1948; Lagerlöf & Werkö 1949). The intermediate pulmonary capillaries may be regarded as a part of the manometric system. Myers & Taylor reasoned that if this could be applied to the more complicated circulatory system of the liver, the pressure transmitted via a catheter occluding and thereby arresting the

flow through a small peripheral branch of the hepatic vein would be a measure of the pressure prevalent on the portal side of the liver sinusoids. In other words, the portal pressure — or to be more precise — the presinusoidal pressure would be transmitted via a static column of blood to the occluding catheter, where it could be measured.

Myers & Taylor tried the method on cats and recorded an occluded hepatic vein pressure varying from 6 mm Hg to 13.5 mm Hg (average 8.6 mm Hg). The pressure thus recorded were in good agreement with direct measurements of the tension of the portal vein, which were made almost simultaneously, the average difference being only 0.6 mm Hg. The injection of epinephrine increased the pressure of both veins by a roughly equal amount, and ligation of the hepatic artery produced no significant reduction in the pressure of either vein. They also tried the method on 7 patients with portal hypertension caused by cirrhosis of the liver and on 12 normal controls.

Friedman & Weiner (1951) estimated hepatic sinusoid pressure in dogs as the midpoint between pressures measured simultaneously within an occluded hepatic vein tributary and an occluded portal vein branch. The average value of sinusoid pressure so obtained was 11.5 cm $H_2O$, when the average portal vein pressure was 13 cm $H_2O$. The occlusive hepatic vein pressure was found to be constantly higher — by a few centimeters of water — than that in the free portal vein. They concluded that, in view of this rather constant relationship, an occluding hepatic vein catheter may be useful in estimating portal pressure in intact subjects. — Myers & Taylor's preliminary investigations of a few cases of cirrhosis of the liver suggest that the method can be used even for estimation of portal hypertension in man.

Though severe portal hypertension is usually readily recognised by the symptoms it produces, the availability of a method for accurate determination of portal tension must surely be not only of academic interest but also of practical value especially with regard to the possibility of surgical treatment. The method was therefore tried at this department. The purpose of the present paper is to report our experience with the use of this method. A preliminary report was presented on Nov. 24, 1951 (Krook 1952).

## MATERIAL AND METHOD

The series consisted of 14 patients with liver disease, 10 of whom had cirrhosis, 4 patients with congestive heart failure and 10 without symptoms or signs of hepatic disease.

The patients were examined in the post absorptive state. A Cournand catheter, usually No. 8 or 9, was inserted into a superficial cubital vein. Under fluoroscopic control the catheter was passed via the right atrium and the vena cava inferior out into an hepatic vein, usually one on the right side, where it was passed until it occluded the vein. The occluded hepatic vein pressure was then measured, after which the catheter was withdrawn until the tip no longer blocked the vein, and the pressure of the now unoccluded hepatic vein was recorded. Recordings were also made of the pressure prevalent at the time in the right atrium and sometimes of that in the vena cava inferior.

The pressures were measured with the electric condensor manometer of Tybjerg-Hansen and Warburg (1949). The projected level of the right auricle (5 cm below the angulus Ludowici) was taken as base-line. Electrocardiagrams were traced simultaneously with the pressure curves.

Table I. *Pressures (mm Hg) in an occluded and free hepatic vein and in right auricle in 10 controls, 5 cases of congestive heart failure and 14 cases of liver disease.*

| | Controls | | Congestive heart failure | | Liver disease | | | |
|---|---|---|---|---|---|---|---|---|
| | 10 cases without evidence of hepatic disease | | 5 cases (3 constrictive pericarditis, 2 mitral stenosis) | | Liver cirrhosis, 10 cases with clinical signs of portal hypertension | | Hepatit. subchron. (2 cases) and Hemochromatosis (2 cases) without clinical signs of portal hypertension | |
| | Average | Range | Average | Range | Average | Range | Average | Range |
| Occluded hepatic vein ..... | 6.4 | 3—10 | 15 | 11—20 | 21 | 12—27 | 11 | 10—12 |
| Free hepatic vein ..... | 4 | 2—6 | 14.4 | 11—18 | 4.8 | 2—7 | 3 5 | 2—6 |
| Mean difference occluded — free hepatic vein pressures | 2.4 | —1—+6 | 0.6 | 0—2 | 15.4 | 8—22 | 7.8 | 6—10 |
| Right auricle ..... | 2.1 | 0—4 | 14.4 | 11—20 | 1.7 | —2—+5 | 1 | 0—3 |

## RESULTS

Table I summarizes the recordings made in 10 subjects without evidence of hepatic disease, 5 cases of congestive heart failure, 10 cases with liver cirrhosis and clinical signs of portal hypertension and 4 cases of liver disease without such symptoms.

Most of the patients in the control group had valvular heart disease but none of them showed any signs of congestive heart failure — the pressure in the right atrium was normal in all cases. The measurements were made in connection with catheterization of the heart. The occlusive hepatic vein pressure was on the average 6.4 mm Hg (range 3—10 mm Hg) and the pressure in a free unoccleded hepatic vein was 4 mm Hg (range 2—6 mm Hg). The average difference between these pressures was thus 2.4 mm Hg. In their 12 normal cases, Myers and Taylor found an occlusive hepatic vein pressure of 4.8 mm Hg on the average.

These values are consistent with those recorded by direct measurements of portal tension at laparotomy of patients showing no evidence of hepatic disease. *E. g.* Blakemore (1946) reported a portal pressure of 10 cm $H_2O$ (cit. Lichtman 1948). Walker (1952) recorded a portal tension of 8.5—15 cm $H_2O$ in 20 subjects and considered the upper normal limit to be 15 cm $H_2O$. Gray (1951) sets the upper limit at 21 cm $H_2O$.

The measurements made in 5 patients with congestive heart failure are also summarized in Table I. Of these, 2 had mitral stenosis with chronic right ventricular failure, of whom one had chronic ascites, and 3 had constrictive pericarditis with ascites.

The increased pressure in the right atrium impairs venous return and thereby increases the hepatic and portal vein pressures. In such cases of portal hypertension the obstruction may be regarded as post-hepatic in

Table II. *Mean pressures in hepatic vein and v. cava inf. and right auricle in 10 patients with liver cirrhosis and clinical signs of portal hypertension.*

| Name | Case no. | Pressures mm Hg | | | | | Oesophageal varices | Splenomegaly | Ascites | Diagnosis |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Occluded hepatic vein pressure | Free hepatic vein pressure | Mean difference occluded-free hepatic vein pr. | V. cava inf. | Right auricle | | | | |
| S. O. | 16 | 24 | 2 | 22 | 7 | − 2 | − | + | + | Cirrhosis hepatis. |
| A. J. | 17 | 24 | 7 | 17 | - | 5 | + | + | − | » » |
| O. A. | 18 | 17 | 2 | 15 | 5 | 2 | − | + | − | » » |
| H. O. | 19 | 18 | 7 | 11 | 5 | - | + | + | − | » » |
| A. Ö. | 20 | 17 | 4 | 13 | 4 | 3 | + | o | − | Cirrhosis hepatis with spleno-renal end-to-side anastomosis. |
| A. A. | 21 | 27 | - | - | 8 | - | + | + | + | Cirrhosis hepatis. |
| P. B. | 22 | 25 | 7 | 18 | 3 | o | + | + | + | » » |
| H. N. | 23 | 24 | 5 | 19 | 8 | o | − | + | + | » » |
| H. A. | 24 | 12 | 4 | 8 | 4 | 4 | + | + | − | » » |
| L. A. | 25 | (21) | (2) | (19) | (5) | (2) | + | + | − | Cirrhosis hepatis with portacaval end-to-side-anastomosis. |
| Average .... | | 21 | 4.8 | 15.4 | 5.5 | 1.7 | | | | |

contradistinction to the intrahepatic obstruction in liver cirrhosis. In cases of severe prolonged passive congestion of the liver such as in chronic constrictive pericarditis, fibrosis of the liver, however, is liable to occur. But in these 3 cases of advanced constrictive pericarditis there was no significant difference between the occlusive and free hepatic vein pressures. These findings suggest that the liver fibrosis in these cases — unlike Laennecs cirrhosis — do not cause any intrahepatic obstruction to the blood flow.

Table II summarizes the measurements made in 10 patients with liver cirrhosis. All of them showed clinical symptoms of portal hypertension with radiographic evidence of oesophageal varices and/or ascites and/or splenomegaly. The occluded hepatic vein pressure varied between 12 mm Hg and 27 mm Hg (average 21 mm Hg) and the corresponding values in the free, unoccluded he-

tic vein were 2 mm Hg and 7 mm Hg verage 4.8 mm Hg), thus a difference of .4 mm Hg on the average. In these cases liver cirrhosis, portal hypertension is due obstruction of the intrahepatic branches the portal vein, so that the difference tween the portal pressure and the free patic vein pressure was greater than in the ntrols and than in cases in which the portal rtension was due to posthepatic obruction.

In the 4 cases (Table I) of chronic hepatic sease without symptoms of portal hypernsion — no oesophageal varices, ascites or lenomegaly — (2 cases of subchronic hetitis and 2 of hemochromatosis) the pres-re in the occluded hepatic vein was 10 to mm Hg (average 11 mm Hg) and the ee hepatic vein pressure was on the average 5 mm Hg. The occluded hepatic vein pres-re measured in these cases was lower than at recorded in the other cirrhotic cases but ightly higher than that in the controls. hough the lowest value in the cirrhotic tients bordered the highest level measured the controls, in no instance did the values verlap. Myers & Taylor, in their 7 cases liver cirrhosis, found the occluded hepatic ein pressure to be 11.5—32 mm Hg verage 20.2 mm Hg) and the difference tween the occlusive and free hepatic vein ressure was 14.4 mm Hg. Our figures are good agreement with those recorded in eir series of cases.

In patients with portal hypertension, the clusive hepatic vein pressure coincides irly well with the portal pressure measureents made at the operating table. In 18 tients with symptoms of severe portal hypertension, Blakemore (1951) measured a portal pressure of 18 mm Hg to 45 mm Hg before the establishment of a portacaval shunt, and in 10 patients in whom a portacaval shunt was established because of liver cirrhosis Pattison (1949) measured a portal pressure of 18—44 mm Hg. The main indication for surgical intervention in these cases was hemorrhagic oesophageal varices. Walker (1952) reported a portal pressure of 13 to 36.5 cm $H_2O$ (average 23 cm $H_2O$) in 7 patients with liver cirrhosis and ascites, and 20 to 56 cm $H_2O$ (average 36 cm $H_2O$) in 21 patients with liver cirrhosis and a history of hemorrhagic oesophageal varices. In the present series this complication did not occur; this explains why the portal tension in our cases was on the average lower than in the more selected series of Blakemore, of Pattison and of Walker.

Four cases of special interest in the present series are discussed below.

*Case 20.* Man, aged 21. Posthepatitic cirrhosis. Oesophageal varices for which splenectomy and splenorenal end-to-side anastomosis was performed. The splenic vein pressure measured at the operating table just before the establishment of the shunt was 26 mm Hg. After operation the oesophageal varices became less pronounced. Occluded hepatic vein pressure postoperatively was 17 mm Hg. As is clear from Blakemore's series, a certain degree of portal hypertension must be expected even after technically successful operations.

*Case 25.* Man, aged 54. Cirrhosis of the liver. Bleeding oesophageal varices for which portacaval end-to-side anastomosis was performed. In this case, then, no portal blood flowed into the liver, so that the occluded hepatic vein pressure measured must have been transmitted from the hepatic artery, this vessel being the only one through which the blood could flow into the hepatic sinusoids. The pressure

measured on various occasions, was regularly 21 mm Hg. It is difficult to explain why the pressure persisted at this particular level. The pressure was approximately equal to that of the capillary filtration pressure; this might theoretically explain at least a temporary arrest of the pressure at this level. Much higher occlusive hepatic vein pressures, however, have been reported (Myers & Taylor) in cases of portal hypertension and were observed in the present series too.

*Case 23.* Man, aged 52, with alcoholic cirrhosis, ascites and splenomegaly. The hepatic artery was ligated. Portal pressure, as measured in the portal vein at the operating table just before ligation of the artery, was 40 cm $H_2O$ *i. e.* about 29 mm Hg. The occluded hepatic vein pressure measured 4 weeks before operation was 24 mm Hg.

*Case 24.* Woman, aged 66, with liver cirrhosis and oesophageal varices and splenomegaly as well as signs of hypersplenism. The occluded hepatic vein pressure was 12 mm Hg, an extremely low level for such a clinical picture. However, the patient's history helps to explain this. The woman was known to have had liver cirrhosis since 1938, when she sustained her initial attack of hemorrhage from oesophageal varices. She had further attacks in 1942 and 1944. In 1942 splenomegaly with leukopenia was observed. Although both of these complications persisted, splenomegaly has decreased in severity since 1944 and is now only radiographically demonstrable. Radiograms still show evidence of oesophageal varices, and laboratory findings still indicate hepatic disease, but the patient feels well and is carrying on with her usual work. Her blood pressure is 250/140 mm Hg, which is remarkable because arterial hypertension is said to be rare in cases of long-standing liver cirrhosis. This is ascribed to the depressant effect of V. D. M. (Vaso depressor material), which according to Shorr (1947) is produced in increased quantities by the cirrhotic liver. It is obvious that this patient had had severe portal hypertension that had caused oesophageal bleeding on various occasions. With the gradual development of collaterals, and possibly with a gradual arrest of the cirrhotic process, the portal tension decreased and the patient improved.

## DISCUSSION

In the normal liver the hepatic sinusoid are the only anastomoses between the hepati vein on the one hand and the portal vei and the hepatic artery on the other hanc Nothing is known about the mechanisr equalizing the high pressure of the hepati artery and the low pressure of the port vein at the site of their common entry int the hepatic sinusoids or in other presinu oidal communications between the two sy stems (Mac Indoe 1928). Pressures re corded via a catheter effectively occluding peripheral branch of the hepatic vein mu have been transmitted via the hepatic sinu oids, these being the only communicatior between the occluded vessel and the pr sinusoidal vessels. That the hepatic arter pressure does not normally influence th pressure measured in an occluded hepat vein has been shown by Myers & Tayl (1951), who found that ligation of th hepatic artery of cats produced no sign ficant fall in the presure of the portal ve or of an occluded hepatic vein.

In the cirrhotic liver, however, the ci culatory conditions are quite different. Th first "portal shunting phase" results in dive sion of portal blood only, which is shunt away via the collateral channels befo reaching the sinusoids of the parenchym and finally portal blood would be total excluded from the sinusoids and hence al from the liver cells, which has been demo strated by MacIndoe (1928). Some write such as Elias, Petty & Popper (1951) cla to have observed anastomoses between fai

rge branches of the portal vein and of the epatic vein — a phenomen which Moschwitz (1948) terms angiogenesis. This ould mean that part of the portal blood ight be shunted past the parenchyma via ese intrahepatic Eck-fistulae immediately ver into branches of the hepatic vein. If ich anastomoses really occur and communite with the hepatic vein branches perineral to the occluding tip of the catheter, would mean a more direct transmission f the portal blood pressure to the catheter.

As fibrosis proceeds, the disease enters a econd, arterial shunting phase in which increasing resistance to arterial blood flow esults in an increasing diversion of arterial lood through presinusoidal arteriovenous unts directly into the portal circuit. Thus, he pressure of the hepatic artery is able to influence the portal pressure via these rteriovenous shunts, which has been demonrated in experimental studies by Berman, Koenig & Muller (1951) and in perfusion xperiments by Herrick (1907), Mac Indoe 1928) & Dock (1942).

The circulation in the cirrhotic liver, in hich the vascular pattern is severely disturbed, is not yet properly understood. Hepatic nsufficiency in liver cirrhosis is probably scribable more to a disorder of the hepatic irculation than to the destruction of hepatic ells, with their high power of regeneration. t is therefore important, especially in the hoice of a suitable therapy, to obtain better nowledge of the pathologically disturbed epatic circulation in cirrhosis, *i. e.* of the echanism of portal hypertension and its elation to the arterial flow into the liver nd to the venous obstruction and of the extent and importance of intrahepatic arteriovenous communications.

If the method described permits true measurements of the portal pressure, as it apparently does, although this cannot be confirmed until after direct simultaneous measurement of the portal pressure at the operating table, it would facilitate the study of various problems bearing on the etiology of portal hypertension. Investigation of a large series of cirrhotic cases would probably elucidate several aspects of portal hypertension. In hospitals with the necessary equipment and team working on heart catheterization, the examination is fairly simple and the patient does not incur any undue risks.

## SUMMARY

A new method recently described by Myers & Taylor for measuring portal pressure by occlusive catheterization of a hepatic vein was tried on (a) 10 persons showing no symptoms or signs of hepatic disease, on (b) 14 patients with chronic disease of the liver, of whom 10 had liver cirrhosis and portal hypertension syndrome and on (c) 5 patients with congestive heart failure.

a) The occluded hepatic vein pressure recorded in those without apparent liver disease ranged from 3 mm Hg to 10 mm Hg (average 6.4 mm Hg).

b) In the group with cirrhosis, the occluded hepatic vein pressure was increased, the increase usually varying with the severity of clinical symptoms of portal hypertension. In the 10 patients with symptoms of portal hypertension the pressure recorded varied from 12 mm Hg to 27 mm Hg (average 21 mm Hg).

c) In the 5 patients with congestive heart failure, 3 of whom had constrictive pericarditis, the level was about the same as that of the systemic vein pressure, *i. e.* 11 mm Hg —20 mm Hg (average 15 mm Hg).

The pressure recorded in a central, unoccluded hepatic vein was low both in apparently healthy persons and patients with cirrhosis, the averages being 4 mm Hg and 4.8 mm Hg respectively.

Much suggests that the pressure measured via a catheter occluding a small peripheral branch of the hepatic vein represents the pressure in the portal vein, and that the method is useful for assessing the degree of portal hypertension.

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# AN ARRANGEMENT FOR CLEANING PIPETTES

By J. LUNDIN

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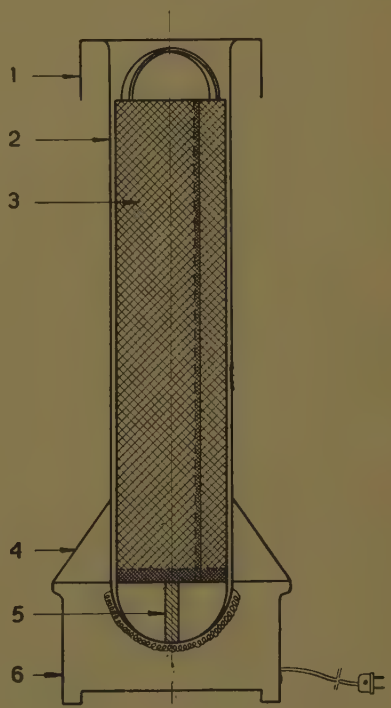
In biochemical, bacteriological and certain ysiological work it is often not sufficient clean the pipettes in water only, but one s to use strong dissolving chemicals such cold sulphuric acid with potassium bis- romate or alkaline potassium perman- nate. However, not even these are quite tisfactory, especially when working with ids and proteins. On the other hand, warm lphuric acid with potassium bischromate s had a good effect in cleaning pipettes. ccording to this an arrangement has been ade for washing pipettes comfortably in arm sulphuric acid with potassium bis- romate.

A big glass cylinder of Pyrex (French) s melted round in one end, then being cm long and having a diameter of 10.5 .[1] (2) By means of a stand the tube was ked upright in an Electrothermal Heating antle, Type 104, Size 1/2 l. (6) In the ver round end of the tube was placed a pport (5) for a pipette basket made of inless steel net. The basket measured × 9 cm and was supplied with a handle. ) Round the tube was placed a mantle ) of stainless steel to protect the Heating antle from drops of acid. The tube open- g was covered when the apparatus was not ed (1). The material for support, the ntle and the handle were stainless steel 3. The net was Sandvik 2R3. The tube was filled with the solution of potassium bio- chromate in sulphuric acid, the handle of the basket being left free over the surface.

When washing the dirty pipettes, they are placed in the basket which thereupon is




[1] The glasstube has been manufactured by dolph Grave Ltd., Stockholm, at a prize of ut $ 6.

lowered in the tube. The Heating Mantle is switched on. A thermometer shows when the temperature has reached 60 or 65° C. This will take about 2 hours with this arrangement. The basket with the pipettes is then lifted over into an automatic pipette washer, where they are allowed to remain for 15 or 20 hours (overnight). After having been rinsed also in distilled water the pipettes are dried in alcohol and ether or in acetone, sucked through by water suction. They can also be dried in air not warmer than 60° C.

To control pipettes washed in the way described above their assigned volumes have been weighed before and after washing. The volumes thereby have been found to vary less than ± 5 per mil, which variation ought to be allowed in most work here concerned. As the volumes of the pipettes vary more with increasing temperature, and as the cleansing property of the acid is good at 60° C, it has not been considered necessar to use still warmer acid.

Thanks to the long rinsing time in runnin water there has been no trouble due to pos sibly adsorbed chromate ions, at least i work with cholinesterase and urease.

The stainless steel parts have been ur corroded by the acid after 4 months' use.

As to the heating arrangement, it certainl could be made considerably cheaper by usin a simple resistance wire isolated by gypsum the wire being adjusted so as to give no more than 60° C in the acid, all preferabl made in the laboratory.

I hope this simple construction will co tribute to solve a small but still rather irr tating problem in laboratory work.

ACKNOWLEDGEMENT

The author wishes to express his thanks to th chief of this institute, Professor Gustaf Ljunggre for his kind interest in this work.

# RAPID STAINING OF SPERMATOZOA

By L. PAULSEN and J. VARNEK

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(Received for publication March 10, 1953)

Many of the staining methods used for purpose of morphological examination semen specimens are beset with the disvantage that they are very time-consumg, often taking up to one hour (Hammen-44, Olesen 1948). A staining method reiring only ten minutes has been reported previous studies (Varnek 1941, 1944). tisfactory results were obtained by this thod in more than 500 cases. The techque employed involved staining with carfuchsin-eosin and after-staining with thylene blue. In order to obtain a simpler d more rapid method the technique has w been modified on this point (Varnek). The technique now adopted is as follows:

(1) Spread one drop of semen on a slide, which has previously been cleaned in alcohol and passed through a Bunsen flame.
(2) Pass the dried smear slowly through a Bunsen flame three times.
(3) Fix in a 1 per cent chloramine solution for 2 minutes.
(4) Rinse cautiously by dipping the slide once into distilled water.
(5) Fix in absolute alcohol for 1/2 minute.
(6) Dry the slide gently with filter paper.
(7) Stain with *carbolgentian violet* for 3 minutes.
(8) Rinse in distilled water and blot gently with filter paper.
(9) Apply one drop of immersion oil, and the specimen is now ready for microscopic examination.

The formula of the carbolgentian violet solution is:

| | |
|---|---|
| Gentian violet | 50 cg |
| Phenol | 5 g |
| Glycerin | 50 g |
| Distilled water, to make | 100 g |

This method yields a fine violet blue staining of the heads and tails of the spermatozoa, whereas the protoplasmic elements take a lighter violet stain. The anterior end of the spermatozoal heads stains less deeply than the basal part. We have used this method in 100 cases and find that it gives beautiful specimens.

## SUMMARY

A method for rapid staining of spermatozoa with carbolgentian violet is reported. The procedure takes 8 minutes.

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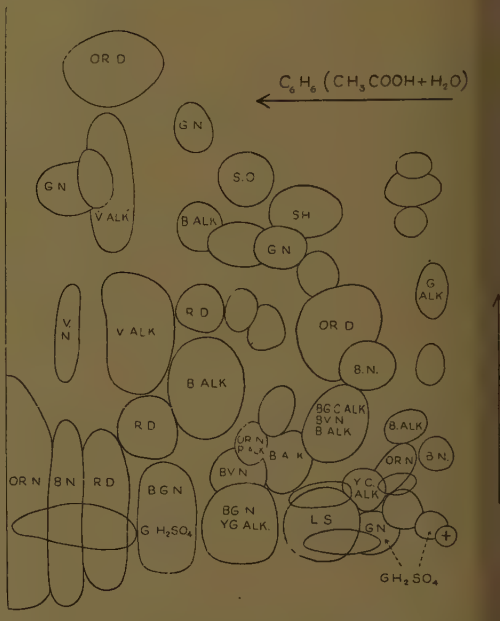
# ACIDIC AND PHENOLIC COMPOUNDS IN HYDROLYSED HUMAN URINE, WITH PARTICULAR REFERENCE TO DISORDERS OF THE LIVER

The observation of Aterman & Boscott (1950) that an abnormal phenolic compound occurred in the urine of patients with liver disease has been extended to the study of 130 normal hospitalised individuals with a variety of clinical disorders. Fraction III (Boscott, 1952 a) of acid hydrolysed urines has been studied by paper chromatographic methods which have been reported briefly elsewhere. (Boscott, 1952 a, b.) The present results confirm the earlier observation that a phenolic compound now provisionally designated "LS" is present in the urine of patients with proved liver dysfunction, but is absent from the urine of almost all apparently normal subjects. Although LS has always been found in the urine of patients with abnormal liver function tests it was also detected in the urine of patients with suspected liver damage, such as occurs in cases of hepatolenticular degeneration, in which the commonly used liver function tests were however, normal.

Clinical evidence to be published elsewhere suggests that the occurrence of LS in the urine is a sensitive index of a metabolic derangement which is not always accompanied by structural damage in the liver. Thus compound LS was present in the urine of a high proportion of patients with hyperthyroidism, steatorrhoea, toxaemia of pregnancy, rheumatoid arthritis and refractory megaloblastic anaemia.

The position of compound LS relative to the distribution of other components on a paper chromatogram of Fraction III from the urine of a patient with proven fibrosis of the liver is shown in the accompanying figure




*Key.* Compounds detected in Fraction III (fro patients with fibrosis of the liver).

B.N, B.G.N, B.V.N, G.N, O.R.N: blue, blue-gree blue-violet, green and orange fluorescences neutral pH.

B.ALK, G.ALK, R.ALK, V.ALK, YG.ALK: blu green, red, violet, and yellow-green fluo escences with 10 per cent $Na_2CO_3$ solution.

BG.C.ALK, YC.ALK: blue-green and yello colours with 10 per cent $Na_2CO_3$ solution.

$GH_2SO_4$: green fluorescence with 90 per cent $H_2SO_4$.

S.O.: compound detected by intense sweet odo

SH: shadow (background fluorescence quenche

ORD, RD, LS: orange, red and salmon-oran azo dyes formed by coupling with diazotis I.C.I. 5091 in 10 per cent $Na_2CO_3$ solution. Remaining compounds were found in oth urines.

⊕ Starting position.

ith descriptive key. In general, fewer components and smaller amounts of those present ere encountered in the urines of normal dividuals and of patients who did not crete LS.

The identity of LS is at present unknown. n absolute alcohol it shows an absorption aximum close to 290 m$\mu$, it gives a positive eaction with the Folin-Ciocalteu reagent but does not react with Millon's reagent, 2:4 dinitrophenylhydrazine hydrochloride, ammoniacal silver nitrate, ninhydrin, and Ehrlich's reagent. LS does not fluoresce in ultraviolet light Wood's glass filter) nor does it give colour or fluorescence reactions with 90 per c ent v/v sulphuric acid, but couples readily with diazotised aromatic amines in alkaline solution.

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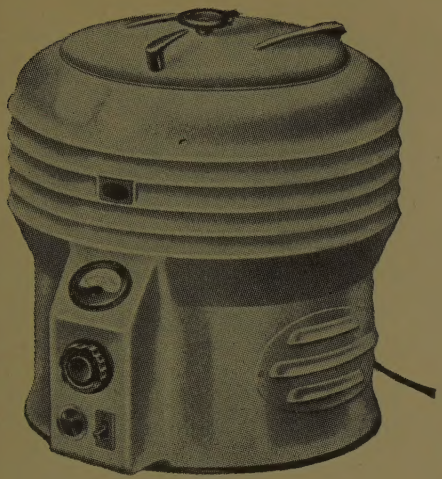

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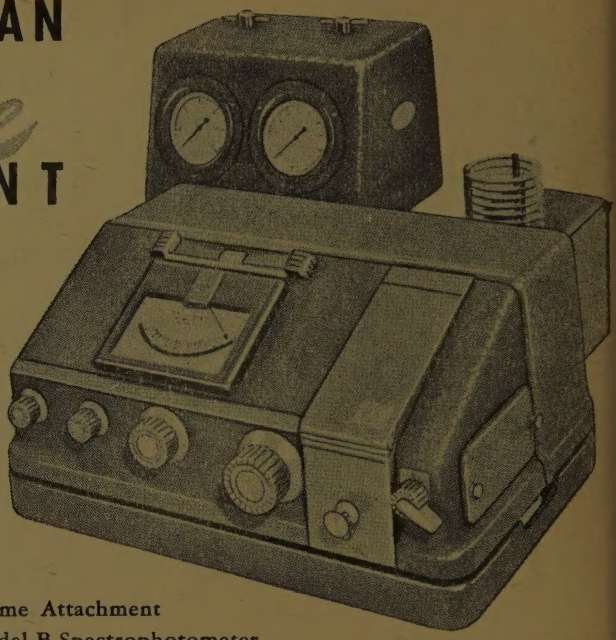

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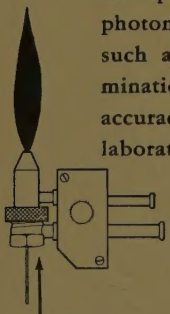

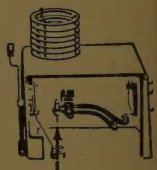

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# CONTENTS

S. TIMONEN AND K. A. SCHRODERUS: The effect of aneurin on uterine contractions ..... 207
K. G. PAUL: Separation of porphyrins by means of counter-current distribution 212
J. GOA: A micro biuret method for protein determination ..... 218
F. RAASCHOU AND K. TRAUTNER: Obstruction of the common bile duct in experimental renal hypertension in dogs ..... 223
A. GRÜNER AND T. HILDEN: The occurrence of chylomicrons in the blood in young and old individuals ..... 236
A. GRÜNER, K. HILDEN AND T. HILDEN: The effect of heparin and protamine sulphate on the occurrence of chylomicrons in human blood ..... 241
V. LEPPÄNEN AND N. HALLMAN: Improved instrument for internal standard flame photometry ..... 250
S. J. DENCKER: Studies in artificial peripheral embolism in the rabbit ..... 261
L.-E. TAMMELIN: An electrometric method for the determination of cholinesterase activity. I ..... 267
H. DIAMANT AND L.-E. TAMMELIN: An electrometric method for the determination of cholinesterase activity. II ..... 271
E. PRÆTORIUS AND H. POULSEN: Enzymatic determination of uric acid ..... 273
G. Y. KENNEDY: Partition chromatography of the porphyrins ..... 281
H. KROOK: Estimation of portal venous pressure by occlusive hepatic vein catheterization ..... 285

## Technique and practical problems

J. LUNDIN: An arrangement for cleaning pipettes ..... 293
L. PAULSEN AND J. VARNEK: Rapid staining of spermatozoa ..... 295

## Letters to the editors

K. ATERMAN, R. J. BOSCOTT AND W. T. COOKE: Acidic and phenolic compounds in hydrolysed human urine, with particular reference to disorders of the liver 296

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